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PLANT PHYSIOLOGY

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KINETICS OF AUXIN INTERACTION

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Received February 19, 1953

Introduction

The purpose of the present work is to examine the interaction of chemically different auxins in their role as agents for the promotion of plant growth. It is proposed to study experimentally the conditions under which one auxin may influence the action of a second chemically different auxin and to determine the principles according to which this interaction occurs. An auxin will be defined for the present purposes as a chemical substance capable of eliciting certain growth responses in plant tissue. Among these growth responses, that of cell elongation is perhaps the most striking and characteristic. The experimental work on which this discussion is based has all been done using elongation of excised oat coleoptile sections as the measure of physiological activity.

To the auxin concept may be coupled that of the antiauxin. An antiauxin will be defined as a chemical substance not itself possessed of growth-promoting activity but with the ability to inhibit competitively the action of auxins (**4**). The present discussion does not however have to do with the inhibition of auxin effects by auxin antagonists. It is concerned rather with the interaction between compounds which themselves possess auxin activity. Perhaps the first detailed study of this kind was that of SKOOG *et al.* (**12**) who investigated the effect of γ-phenylbutyric acid upon indoleacetic-acid-induced growth of the Avena coleoptile. γ-Phenylbutyric acid is itself an auxin although its activity in the Avena curvature test is only 1×10^{-5} that of indoleacetic acid. Although the growth-promoting activity of γ-phenylbutyric acid in the Avena coleoptile section test is so small as to have escaped SKOOG *et al.* (**12**), such activity has been found by MUIR *et al.* (**11**) and has been confirmed in the present study. γ-Phenylbutyric acid is not only less active than is indoleacetic acid in the Avena section test but is in addition active only over a smaller concentration range.

The inhibition of the action of indoleacetic acid by γ-phenylbutyric acid reported by SKOOG *et al.* (**12**) may be shown by appropriate treatment to be a competitive one (**4**). It appears then that an active auxin may be com-

petitively inhibited in its effect within the plant by other auxins of lesser activity. Observations generally similar to those made concerning the action of γ-phenylbutyric acid have since been made by THIMANN and BONNER (**13**) on triiodobenzoic acid. Phenoxyacetic acid which possesses but little activity as an auxin has similarly been shown to antagonize the action of more active auxins. Thus ÅBERG (**2**) found this compound effective in counteracting inhibition of the growth of flax roots induced by 2,4-dichlorophenoxyacetic acid while AUDUS and SHIPTON (**3**) have shown that it relieves inhibition of cress root growth that is induced by 2,4-dichlorophenoxyacetic acid.

It would seem reasonable to suppose in a qualitative way that the action of a more active auxin might be inhibited by a less active auxin through some sort of competition for the site within the plant to which auxins become attached in order to exert their effect. This supposition would imply not only that auxins become attached in the plant but also that diverse auxins exert their effects through attachment at a common site. That these suppositions are correct has been strongly indicated by the fact that any given antiauxin inhibits the effects of many, perhaps of all, different auxins (**1, 9, 14**).

It has been shown in earlier papers (**5, 9**) that the action of auxins in promoting the growth of the Avena coleoptile sections can be formally treated by the methods of classical enzyme kinetics. It has been further shown that the activity of an auxin can be characterized by two parameters which correspond to the K_m and V_{max}, respectively, of MICHAELIS-MENTEN (**10**) kinetics. Thus, the parameter K_m is a measure of the affinity of a given auxin for the auxin-receptive site or point of attachment within the plant. The ability of the complex, once formed to promote growth is measured by the parameter V_{max}. The way in which two auxins interact within the plant to determine growth rate will be shown in the present paper to be dependent upon the value of the constants, K_m and V_{max}, for each of the interacting materials.

Materials and methods

The Avena seedlings (var. Siegeshafer) used in this investigation were germinated in vermiculite and grown for 82 to 84 hours in a standard Avena dark room under weak red light at a temperature of 25 to 26° C and a humidity of 90%. Coleoptiles 2.75 to 3.25 cm. in length were selected for use, the leaves removed and from each coleoptile a single apical section, 5.0 cm. in length, was cut so as to discard 2 to 3 mm. of the tip. The sections were pooled and randomized in lots of 20 between the individual treatments of each experiment. The basal nutrient solution consisted of 3% sucrose, 0.0025 M potassium maleate buffer (pH 4.50) and Pyrex redistilled water. This nutrient, to which the varied auxins were added in the appropriate concentrations, was dispensed into Petri dishes, 20 ml. per dish. The sections, 20 per dish, were allowed to float on the surface of the nutrient during the experimental period. All of the growth measurements here recorded

were made after 12 hours of incubation (or less) and are therefore a measure of initial growth rate since growth under these conditions is linear with time for 18 to 24 hours.

Results

Kinetic treatment of auxin-induced growth

First considered are the experimental relations between auxin concentration and the growth rate of Avena sections. The curves which express this relation (figure 1 A) are similar to the curves which have been found to relate substrate concentration to rate of many enzymatic reactions. The present data on auxin-induced growth have therefore been treated, as in earlier papers (**5, 9**), by the methods of classical enzyme kinetics in order to determine the extent to which these methods are applicable. This treatment is based upon the supposition that auxin becomes bound to some receptive site within the plant and that a ready equilibrium is established between the free and bound forms. It is further supposed that rate of growth is proportional to the concentration of appropriately bound auxin. This situation may be formulated as in equation 1.

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k} \text{GROWTH} \tag{1}$$

The quantity E of equation 1 represents the auxin receptive enzymatic site within the plant; S similarly represents the substrate, in this case auxin, and ES the enzyme-auxin complex or bound auxin. K_s and k are constants of the system. The reaction velocity (growth rate), v, is assumed in classical enzyme kinetics to be proportional to the concentrations of ES, or as expressed by equation 2.

$$v = \frac{k\,[E][S]}{[S] + K_s} = \frac{V_{max}\,[S]}{[S] + K_s} \tag{2}$$

In this equation V_{max} is the rate which will obtain when all available receptive sites are combined with auxin. It must be assumed, as in classical enzyme kinetics, that the concentration of sites is small with respect to the concentration of auxin. That the total concentration of receptive sites remains constant with time is indicated by the fact that the growth rate is constant over the period of measurement. Equation 2 is more conveniently used in the reciprocal form which may be represented graphically by plotting the reciprocal of the initial rate as a function of the reciprocal of the initial substrate (auxin) concentration. The reciprocal equation and data for enzymatic systems which follow it yield a straight line with a slope of K_s/V_{max} and with an intercept of $1/V_{max}$ (**8**).

We can now describe the initial growth rate of the system as a function of auxin concentration by equation 2 and we are now able to determine values for constants V_{max} and K_s, provided that the above assumptions are indeed correct. Figure 1 A shows that for the systems investigated auxin

concentration influences growth of Avena coleoptile sections in a manner consistent with equation 2. Similarly the double reciprocal plots (1/v against 1/[S]) of figure 1 B show that the straight lines obtained are those to be expected from the reciprocal form of equation 2. The values of V_{max} and K_s for each system may be determined graphically from the data in the form presented in figure 1 A but may be evaluated more precisely from figure 1 B. The values for the two parameters obtained from the straight lines of figure 1 B are collected in table I. The values for K_s presented in the table are somewhat larger than those previously reported (**5, 9**) due to the placing of less weight on the low concentration values in which the residual endogenous auxin of the section becomes an important factor.

It may be noted that the quantitative values of K_s as here determined depend not only on the affinity of the receptive site for the auxin in question but also upon the relation of external to internal auxin concentration. The

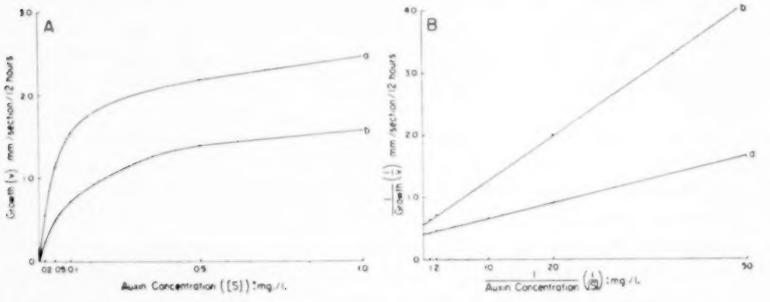

FIG. 1. Auxin-induced Avena coleoptile section growth. A. Growth as a linear function of auxin-concentration. B. The reciprocal of growth as a reciprocal function of auxin concentration. The experimental data used for A and for B are the same. The two curves are those for IAA (a) and for 2,4-D (b).

values relative to naphthalene acetic acid (NAA) which are given in table I have not been included in figure 1 since the K_s and V_{max} are very close to those of 2,4-dichlorophenoxyacetic acid (2,4-D). The parameters of table I emphasize the fact that the synthetic auxins, 2,4-D and NAA, have not only a somewhat lower affinity (larger K_s) than does indoleacetic acid (IAA) for the receptor site of the Avena coleoptile, but also that receptor sites combined with 2,4-D or NAA are less effective in promoting Avena section growth (lower V_{max}) than are such sites combined with IAA.

EQUILIBRIUM CONDITIONS OF AUXIN-INDUCED AVENA SECTION GROWTH

It has been pointed out above that the present treatment presupposes that equilibrium conditions prevail between free auxin and auxin bound to the receptive site within the coleoptile. The validity of this assumption may be readily demonstrated by the results of experiments in which Avena sections are first grown for a short period in a solution containing IAA as the growth substance and then transferred to a solution containing 2,4-D or vice

TABLE I

CONSTANTS WHICH CHARACTERIZE THE ACTION OF CERTAIN AUXINS ON THE GROWTH OF AVENA COLEOPTILE SECTIONS.

Substance	K_s (molar)	V_{max}
		mm./section/12 hrs.
IAA	4.5×10^{-7}	2.6
2,4-D	7.6×10^{-7}	1.7
NAA	7.2×10^{-7}	1.6

versa. Transfer from one solution to the other occurred in each case after a six-hour interval and measurements were made at three-hour intervals for a total of 12 hours. The data of table II and figure 2 show that sections growing at a rate determined by the concentration of IAA in the medium changed this growth rate to that appropriate to the concentration of 2,4-D when transferred to a fresh medium containing this substance as the active component. Transfer in the reverse direction (2,4-D to IAA) yielded similar results. Thus, after transfer of Avena sections from a medium containing IAA to one containing 2,4-D, the receptor sites become equilibrated with the new growth substance. The time needed for consummation of the new equilibrium condition is evidently short as compared to three hours since sections put in, for example, 2,4-D after having been in IAA grow at the same three-hour rate as sections which are continuously in 2,4-D.

Avena section growth in presence of auxin mixtures

It has been shown above that the relation of auxin concentration to the initial growth rate of Avena sections can be treated quantitatively by orthodox chemical kinetics. Let us now consider what may be expected to happen if two chemically different auxins were to be simultaneously supplied to such sections. Suppose, for example, that auxin 1 (concentration S_1) and auxin 2 (concentration S_2), both act at the same site within the plant and that

TABLE II

EFFECT ON GROWTH OF AVENA COLEOPTILES OF TRANSFERRING SECTIONS FROM IAA TO 2,4-D AND VICE VERSA. THE CONCENTRATION OF IAA OR OF 2,4-D USED WAS 2.89×10^{-6} M.

Medium	Time of measurement			
	3 hours	6 hours	9 hours	12 hours
	mm./section/3 hours			
IAA for 12 hrs.	0.68	0.65	0.69	0.65
2,4-D for 12 hrs.	0.45	0.43	0.44	0.44
IAA for 6 hrs. then	0.68	0.65	....	....
2,4-D for 6 hrs.	....	....	0.47	0.43
2,4-D for 6 hrs. then	0.45	0.43	....	....
IAA for 6 hrs	....	....	0.68	0.65

the two free auxins are in equilibrium with the bound forms ES_1, and ES_2, respectively. This situation is equivalent to that described by FOSTER and NIEMANN (**6**) for the competitive interaction of two substrates at a common enzymatic site. For this case the overall reaction velocity (growth rate) may be shown (**6**) to be given by the expression in equation 3.

$$(3) \qquad v = \frac{V_{max_1}\frac{[S_1]}{K_{s_1}}}{1 + \frac{[S_1]}{K_{s_1}} + \frac{[S_2]}{K_{s_2}}} + \frac{V_{max_2}\frac{[S_2]}{K_{s_2}}}{1 + \frac{[S_2]}{K_{s_2}} + \frac{[S_1]}{K_{s_1}}}$$

The quantities V_{max_1} and V_{max_2} of equation 3 represent respectively the individual quantities, V_{max}, for the two substrates S_1 and S_2 while K_{s_1} and K_{s_2} represent the respective affinity constants. The first term of equation 3 gives the growth elicited by auxin 1 while the second term gives the growth due to auxin 2. Thus for a single auxin ($[S_2] = 0$) equation 3 assumes the form of original equation 2. The use of equation 3 is facilitated by its transformation to the reciprocal form. Systems which follow equation 3, must in reciprocal form, and for any given constant ratio of S_1/S_2 be expected to yield a linear relationship between $1/v$ and $1/[S_1] + [S_2]$. From this linear relationship it is then possible to compute the apparent parameters $V_{max\,(combined)}$ and $K_{s\,(combined)}$ for the complex system as a whole. The apparent constants for the two-auxin system are related to the constants derived earlier for the individual systems by equations 4 and 5 below:

$$(4) \qquad V_{max(combined)} = \frac{V_{max_1}\frac{[S_1]}{K_{s_1}} + V_{max_2}\frac{[S_2]}{K_{s_2}}}{\frac{[S_1]}{K_{s_1}} + \frac{[S_2]}{K_{s_2}}}$$

$$(5) \qquad K_{s\,(combined)} = \frac{[S_1] + [S_2]}{\frac{[S_1]}{K_{s_1}} + \frac{[S_2]}{K_{s_2}}}$$

Thus, from a knowledge of the constants V_{max} and K_s which describe the effects of individual auxins, we should be able to predict the over-all effects of combinations of auxins. Conversely by measuring the parameters $V_{max\,(combined)}$ and $K_{s\,(combined)}$ obtained for growth in the presence of an auxin mixture and by comparing these values with those obtained by calculation from the appropriate parameters of single auxin systems we can determine

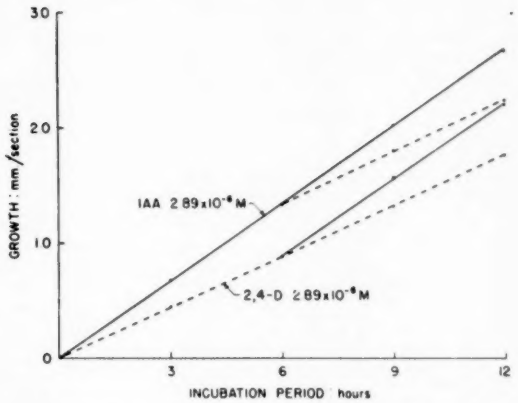

FIG. 2. Avena coleoptile section growth in the presence of IAA (solid line) and of 2,4-D (dashed line). After six hours incubation, sections in IAA were transferred to 2,4-D and vice versa for a further six-hour period.

whether or not the present formulation adequately describes the growth response of Avena sections to auxin mixtures.

That mixtures of auxins do indeed influence growth of Avena sections according to principles here outlined is shown by the data of figure 3. These present the data from experiments on the interaction in Avena section growth

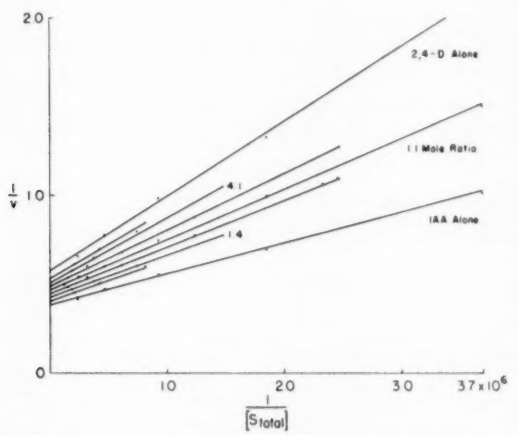

FIG. 3. Interaction in Avena section growth of IAA and 2,4-D given individually or in a variety of concentrations and molar concentration ratios. Data plotted according to the method of FOSTER and NIEMANN (6) as a test for competition at the auxin-receptive site. The upper and lower lines are those obtained for 2,4-D alone and IAA alone, respectively. The family of lines between are, in descending order, those obtained for the following 2,4-D to IAA molar ratios: 8 : 1, 4 : 1, 2 : 1, 1 : 1, 1 : 2, 1 : 4, and 1 : 8.

TABLE III

SUMMARY OF CONSTANTS K_s AND $V_{max.}$ FOR IAA AND 2,4-D ALONE AND IN COMBINATION AT VARYING MOLAR RATIOS.

Per cent. composition in interaction mixture		K_s (molar)		$V_{max.}$	
IAA	2,4-D	Observed	Calculated	Observed	Calculated
				mm./section/12 hrs.	
100	0	$4.6 + 10^{-7}$	....	2.6	....
80	20	5.0×10^{-7}	5.2×10^{-7}	2.4	2.4
67	33	5.3×10^{-7}	5.5×10^{-7}	2.3	2.3
50	50	5.8×10^{-7}	5.9×10^{-7}	2.2	2.2
33	67	6.4×10^{-7}	6.7×10^{-7}	2.0	2.1
20	80	6.8×10^{-7}	7.0×10^{-7}	1.9	1.9
0	100	7.6×10^{-7}	....	1.7	....

of IAA and 2,4-D given alone and in a variety of concentrations and molar concentration ratios. The reciprocal of initial growth rate ($1/v$) is plotted as a function of the reciprocal of total auxin concentration ($1/[S_{total}]$) for each of these combinations. The data for each individual auxin yield a straight line with characteristic slope and intercept as expected from the similar experiments of figure 1 B. The data for the auxin mixtures yield in each case a family of straight lines lying between the lines for the individual auxins as predicted by equation 3. The slope and intercept of each line is determined by the molar concentration ratio between the two auxins. Table III gives a compilation of the values for the constants $K_{s\,(combined)}$ and $V_{max\,(combined)}$ as measured for the several pairs of auxins at various molar ratios and compares these values with those calculated from equations 4 and 5 using the previously determined values of K_s and V_{max} for the individual auxins. It is evident that there are no considerable differences between the measured and calculated values of the two combination parameters although

TABLE IV

SUMMARY OF CONSTANTS K_s AND $V_{max.}$ FOR IAA AND NAA ALONE AND IN COMBINATION AT VARYING MOLAR RATIOS.

Per cent. composition in interaction mixture		K_s (molar)		$V_{max.}$	
IAA	NAA	Observed	Calculated	Observed	Calculated
				mm./section/12 hrs.	
100	0	4.4×10^{-7}	....	2.6	....
80	20	4.8×10^{-7}	4.8×10^{-7}	2.3	2.4
67	33	5.0×10^{-7}	5.1×10^{-7}	2.2	2.3
50	50	5.3×10^{-7}	5.5×10^{-7}	2.1	2.1
33	67	5.8×10^{-7}	5.9×10^{-7}	1.9	2.0
20	80	6.5×10^{-7}	6.4×10^{-7}	1.8	1.8
0	100	7.2×10^{-7}	....	1.6	....

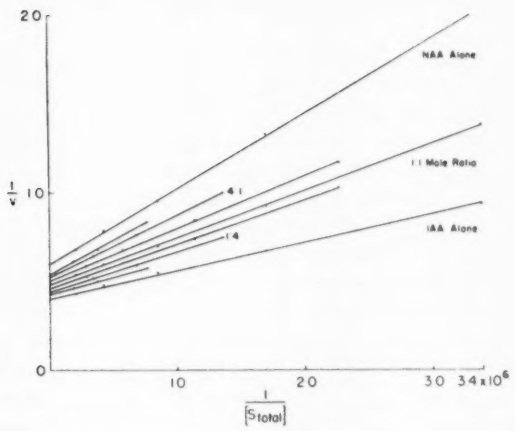

FIG. 4. Interaction in Avena section growth of IAA and NAA given individually or in a variety of concentrations and molar concentration ratios. Data plotted according to the method of FOSTER and NIEMANN (6) as a test for competition at the auxin-receptive site. The upper and lower lines are those obtained for NAA alone and IAA alone, respectively. The family of lines between are, in descending order, those obtained for the following NAA to IAA molar ratios: 8 : 1, 4 : 1, 2 : 1, 1 : 1, 1 : 2, 1 : 4, and 1 : 8.

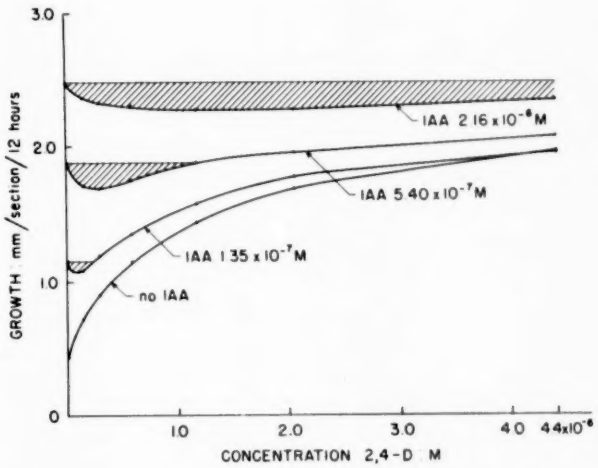

FIG. 5. Avena coleoptile section growth as a linear function of 2,4-D concentration in the presence of various concentrations of IAA. The lowest curve is that obtained in the presence of 2,4-D alone. Each successively higher curve is that obtained in the presence of a higher concentration of IAA. The shaded area of each curve represents the concentration range over which 2,4-D inhibits growth below the level attained in IAA alone.

there is a systematic deviation of the K_s values in favor of IAA of approximately 5%.

Since the K_s and V_{max} values for NAA are approximately the same as those for 2,4-D, it is to be expected that results obtained for the interaction of IAA and NAA should be similar to those found for the interaction of IAA and 2,4-D. That this is so is evident from figure 4 where data obtained for the interaction of IAA and NAA are presented in the appropriate double reciprocal plots. Again, the data for the auxin mixtures yield a family of straight lines which lie between the lines for the individual auxins. Furthermore, it is apparent from table IV that there is close agreement between the observed and calculated parameters. The NAA, therefore, interacts with IAA in Avena section growth in a competitive manner as do IAA and 2,4-D.

Although the interactions between 2,4-D, NAA, and IAA are, as shown above, in general accord with expectations of classical kinetics, there is nonetheless at least one aspect of this interaction which cannot be inter-

TABLE V

THE INTERACTION EFFECT OF IAA AND 2,4-D IN THE GROWTH OF AVENA COLEOPTILE SECTIONS.

Relative concentration of 2,4-D*	Relative concentrations of IAA*						
	0	1	2	4	8	16	32
	mm./section/12 hrs.						
0	0.42	1.12	1.41	1.87	2.19	2.49	2.81
1	0.72	1.09	1.33	1.70	2.06	2.36	2.84
2	0.90	1.20	1.42	1.70	2.09	2.34	2.70
4	1.17	1.37	1.56	1.75	2.06	2.33	2.66
8	1.44	1.58	1.67	1.89	2.09	2.26	2.56
16	1.70	1.79	1.85	1.96	2.11	2.27	2.52
32	1.97	1.96	2.01	2.05	2.17	2.33	2.46

*The relative unit of concentration, $1 = 1.348 \times 10^{-7}$ M.

preted on the present basis. This one aspect is the inhibition of the action of low concentrations of IAA which is elicited by low concentrations of 2,4-D or NAA. That such inhibition occurs is evident from the curves of figure 5 which have been obtained from the data of table V. In this figure, growth rate of the Avena coleoptile section is plotted as a linear function of 2,4-D concentration. Each individual curve is for a system of constant IAA concentration. The shaded area of each curve represents the concentration range over which 2,4-D inhibits growth at a constant IAA concentration. The sharp initial dip in each curve which represents inhibition by low 2,4-D concentrations is scarcely detectable in the double reciprocal plots of figures 3 and 4 which then appear as straight lines. This inhibition is evidently roughly competitive in nature since it is most marked at low IAA concentrations.

Discussion

The experiments described above demonstrate that different auxins interact in the Avena coleoptile section in a manner compatible with the

hypothesis that they react at a common site. They have shown furthermore that the nature of the interaction is dependent upon at least four parameters which are respectively: (a) the K_s for each auxin, (b) the V_{max} for each auxin, and (c) and (d) the concentrations of each auxin. The result of such interaction is that one auxin such as 2,4-D may augment or diminish the effects of a second auxin such as IAA depending on the absolute concentration of each. Thus the data of table V show that 2,4-D acts to diminish the growth response elicited by moderate or high concentrations of IAA. The 2,4-D also acts, in general, to augment the growth response elicited by low concentrations of IAA. The major interaction effects between 2,4-D and IAA are due to the fact that even though 2,4-D has slightly less affinity (higher K_s) for the active site within the plant than does IAA, it also possesses considerably lower intrinsic activity (V_{max}) than does IAA. When 2,4-D is added to a system which contains a concentration of IAA greater than some certain critical value, which is a function of the characteristic parameters of the system ($S_{critical} = K_{s_1} \frac{V_{max_2}}{V_{max_1} - V_{max_2}}$), the 2,4-D replaces IAA at a portion of the available receptor sites. The sites occupied by 2,4-D contribute less to over-all growth rate than do sites occupied by IAA. Even though some previously unoccupied sites may also be occupied by 2,4-D, the over-all effect will nonetheless be to diminish growth rate.

At concentrations of 2,4-D below the critical value, the addition of 2,4-D should result in an increased growth rate because, in this region, there is a relatively large number of unoccupied sites available for combination with 2,4-D. Superimposed upon this effect is however an inhibition which is particularly marked in the case of very low 2,4-D concentrations and which is not predicted by the present treatment.

The qualitative description of the kinetic consequences which result from the presence of 2,4-D in a system which contains IAA applies similarly to the effects of NAA in the presence of IAA. While the interaction of other auxins of low V_{max} with IAA have not yet been so intensively investigated, it may be expected that auxins of low V_{max} will, in general, interact with auxins of higher V_{max} according to general principles here outlined. Competitive interactions of the present type may serve to make understandable such bewildering responses of intact plants to auxin mixtures as those described by HITCHCOCK (7) who applied 2,4-D and/or IAA to tomato plants. The repression of response to 2,4-D by low concentrations of IAA and augmentation of responses to 2,4-D by higher concentrations of IAA recorded by Hitchcock are responses which are consistent with the interactions presented above.

The principles which govern the interaction of two different applied auxins may also be involved in the interaction of an applied exogenous auxin with the native endogenous auxin of the plant. In the pineapple, for example, an interesting anomaly is that synthetic auxins such as 2,4-D or NAA supplied to the plants at low concentrations induce flowering in spite of the fact that flowering under other conditions appears to be associated with a

decrease in the amount of endogenous auxin. BONNER and BANDURSKI (4) have suggested that synthetic auxins may act as antagonists to the native auxin of the pineapple plant. According to this view, the synthetic auxins would at appropriate concentrations compete with the native auxin in such a manner that the effective auxin concentration within the plant would be lowered.

Auxin interactions of this type are to be anticipated on the basis of the present findings and are apparent from the curves of figure 5. In terms of the effects of an exogenous auxin on a plant or plant organ which already contains native IAA, the shaded areas of figure 5 would represent concentration range over which applied 2,4-D will effectively lower the over-all auxin response. It is evident that this type of interaction may account for the induction of flowering in the pineapple plant by applied 2,4-D or NAA provided only that the native auxin of this plant be present in concentrations which do not saturate all of the receptive sites and that flowering is in fact associated with low effective auxin level.

It would appear to be of importance for the understanding of all such auxin interactions that the activity of each auxin be understood and characterized in terms of the parameters K_a and V_{max}.

Summary

It has been found empirically that the promotion of Avena coleoptile section growth by auxin can be treated quantitatively on the basis of the supposition that the auxin molecule exerts its effect through combination with a suitable receptor site within the plant. The growth-promoting activity of an individual species of auxin molecule can be characterized by two quantities, K_a and V_{max}, each of which may be experimentally determined. K_a describes the affinity of the auxin for the receptor site within the plant while V_{max} is the growth response which obtains when all receptive sites are saturated with the particular auxin. The values of K_a and of V_{max} for chemically different auxins are in general different.

Chemically different auxins such as IAA, 2,4-D and NAA compete with one another for the same receptive sites within the plant, as shown by the following steps: (a) formulation of the quantitative relations between growth rate and auxin concentration which would be expected on the basis of competition of auxins for the same sites, (b) derivation of the parameters $K_{a\,(combined)}$ and $V_{max\,(combined)}$ for systems containing two different auxins in terms of the constants K_a and V_{max} for each auxin alone, (c) demonstration that the values $K_{a\,(combined)}$ and $V_{max\,(combined)}$ measured experimentally for growth rate in the presence of auxin mixtures are identical with those calculated on the basis of (b) above.

Auxins of low K_a and low V_{max} are capable of inhibiting or augmenting the activity of auxins of similar K_a and greater V_{max}. Whether inhibition or promotion results depends upon the concentrations of the two substances and upon the differential in V_{max} for the two substances.

The principles according to which chemically different auxins interact in promotion of growth of the Avena coleoptile section appear to be largely understandable in terms of classical enzyme kinetics. Although the correctness of this formulation has been verified only for the Avena coleoptile, these same principles may perhaps be applicable to further plant systems.

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THE DIURNAL VARIATION IN THE TRANSLOCATION OF MINERALS ACROSS BEAN ROOTS

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Present knowledge about the mechanism whereby minerals are transferred from the nutrient medium to the root xylem is quite limited. The initial step involves an exchange of hydrogen, hydroxyl, or bicarbonate ions on the protoplasmic surface of the root for nutrient ions of the substrate (**13**). The amount of any ion gained by such exchange is a function of the rate at which metabolic energy is produced by the root, and, within limits, of the concentration of the ion in the substrate. Absorbed ions appear to enter into labile cytoplasmic complexes which are created or maintained by the expenditure of energy, and which seem to be specific for different ions or groups of related ions.

It is now generally held that the protoplasts of the root cells are interconnected by plasmodesmata forming a symplast of cytoplasm that extends from the root surface to the xylem elements (**3, 5, 11, 20**). Hence, ions entering the cytoplasmic phase by exchange have a pathway through living material to any of the three aqueous phases—external, vacuolar, or tracheal. The cytoplasm in some manner utilizes energy derived from aerobic respiration to speed the distribution of ions throughout the symplast, and to produce high vacuolar and tracheal concentrations of the ions.

There is no convincing evidence as to how movement of ions through the cytoplasm occurs, but several theories and speculations on the matter are current. LUNDEGARDH'S (**11**) theory of an inward anion migration as a countercurrent to an outward flow of electrons, and a cation migration as a series of exchanges for hydrogen ion, has received the most attention, but is now suspect for its failure to explain the action of dinitrophenol, which reduces ion absorption without decreasing respiration (**15**). OVERSTREET and JACOBSON (**14**) have published a review in which they fit the thermodynamic consequences of possible membrane properties to various theories of ion accumulation, and they find support for the view that ion transport through the cytoplasm occurs in conjunction with some ion-carrier, which is presumably formed by aerobic respiration and is removed by some metabolic process after transporting an ion to the inner phase. WIERSUM (**20**) believes that movement through the symplast may be by diffusion along concentration gradients in the intermicellar fluid of the cytoplasm, and by active, inwardly-directed, polar transport of ions bound to the colloidal

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phase. For those who hold that the active transport across the symplast is unidirectional, visualization of some such two-phase transport is essential to an explanation of the finding that ions can move from the stele outward to the external solution (**12, 20**).

The method by which ions are released from the cytoplasm to the vacuoles and xylem elements is as obscure as the means of transport. Conceivably, the release might require the expenditure of energy, and be properly termed secretion; on the other hand, the release may be spontaneous, the requisite energy expenditure for accumulation having been made when the ions were initially taken into the cytoplasm, or the energy may have been expended in the formation of some ion-carrier. The Dutch workers (**1, 3, 20**) seem largely to favor the secretion viewpoint, basing their opinion upon the relative impermeability of the tonoplast to ions resident in the vacuole, and upon the fact that vacuolar or xylem concentrations of ions may sometimes appear to exceed those of the cytoplasm. Most other opinions seem to favor passive release, or at least have the principle of spontaneous release incorporated into the postulated mechanism for absorption and transport. LUNDEGARDH (**11**) now pictures anion-respiration as maintaining a steady-state between active accumulation of salt from the external medium and a passive release to the xylem. He does not believe that it is necessary to visualize a symplast to account for this process; each cell accumulates salts on the outermost side and passively, but selectively, releases them on the inward side. The well-known exudation theory of CRAFTS and BROYER (**7**) incorporates the idea of passive release of ions to the xylem, and BROYER (**6**) also views the release of ions to the vacuole as a spontaneous process. He postulates that ions bound in cytoplasmic complexes at outer loci might migrate to inner phases where some change in metabolic rates or processes would favor release of the ions, increasing the activity of the ions in the intermicellar fluid, and resulting in a spontaneous movement of ions to the vacuole.

It appears to the authors that any distinction between secretion and passive release is rather academic. The cytoplasm cannot perform work beyond its boundaries, and the movement of ions into either vacuole or xylem element must be spontaneous. Regardless of mechanism, the thermodynamic consequences of accumulation require that the cytoplasm expend energy in raising the chemical potential of ions at the inner desorbing surface as compared with that at the outer adsorbing surface. Seemingly, if the mechanism expends energy directly or indirectly at the outer cytoplasmic surface in absorbing ions, the release is to be termed passive; if it expends energy at the inner surface in releasing bound ions, the release is to be termed secretion. Some confusion can be predicted if energy proves necessary for both absorption and release. In this report the term release will refer to the passage of the ions from the cytoplasmic to an aqueous phase and is not otherwise qualified.

These diverse opinions on one of the most fundamental of cytoplasmic mechanisms suggest that what is most needed at the present is more infor-

mation about variables which affect the process of transfer and release of ions to the vacuoles and xylem. It has occurred to the authors that useful information might be obtained from studies of the fate of tracer ions that have entered the root cytoplasm. Depending on the state of the cytoplasmic transfer and release mechanism at any instant, there should be certain probabilities that any one ion would remain with the protoplasm, pass into a vacuole, be deposited in the xylem, or even return to the external medium. If these probabilities can be altered by varying the temperature, oxygen tension, sugar supply to the root, functioning of enzyme systems, salt status, auxin supply, light, transpiration rate, or any other variable which past research has suggested might be concerned in the transfer and release mechanism, it is not unlikely that an extended estimate of the nature of the mechanism can be made.

It appears, however, that before any extensive investigations could be undertaken, some basic information was needed on the ability of the symplast to transport ions to the xylem. Studies of the diurnal fluctuation in exudation (**17**) indicate that there might be a diurnal variation in the capacity of the root symplast to absorb and/or translocate ions. If such is the case, all short-term experiments need to be planned for a specific time of day. HOAGLAND (**10**) and BROYER (**5**) have reported that low-salt, high-sugar barley roots initially transport a smaller proportion of absorbed potassium and bromide to the xylem than do high-salt, low-sugar roots. Presumably, in the more active low-salt roots a greater proportion of the ion being transported inward through the symplast is released to the vacuoles; as the ion concentration of the vacuoles increases, more and more of the absorbed ion reaches the xylem. EATON and JOHAM (**8**) found a positive correlation between the sugar content and the mineral content of cotton roots. Collectively, these findings indicate that the salt and sugar content of roots could have a large effect on the relative amounts of absorbed ion retained in the root, and should be taken into consideration in the culture of experimental plants. Accordingly, some rather simple experiments were performed to determine if there actually is a diurnal fluctuation in the amount of ion absorbed or translocated to the xylem, and the influence of sugar content and salt status on such variation. The 24-hour day was divided into short absorption periods, and by means of radioactive isotopes the distribution of absorbed ion between root and shoot at the end of each period was ascertained.

Methods and materials

Red Mexican bean (*Phaseolus vulgaris*) seeds were germinated under fluorescent lights, and selected seedlings were placed in notched covers over enameled pans containing six liters of a dilute Hoagland's solution (0.00167 M KNO_3, 0.00167 M $Ca(NO_3)_2$, 0.00067 M $MgSO_4$, 0.00017 M KH_2PO_4, micronutrients and iron), six plants per pan. The solutions were aerated and the plants grown for 10 days under banks of fluorescent lamps on a 6:00 A.M. to 6:00 P.M. day, which was time-clock controlled. The light

intensity on the surface of the upper leaves was about 1000 foot-candles. A temperature of 73 ± 1° F and a relative humidity of 60 ± 4% were maintained in the growth room by means of an air-conditioning unit. The plants were always in complete darkness during the night period except for brief exposures to light from an incandescent bulb at the beginning and end of experimental absorption periods.

When low salt plants were desired no change of nutrient solution was made. High salt plants were obtained by placing the plants in full strength Hoagland's solution on the fourth day and by changing solutions on the seventh day. Previous study had indicated that both the growth and the salt requirement of the plants were less than for similar plants raised in full sunlight.

Radioactive isotopes of rubidium (Rb^{86}) and phosphorus (P^{32}) were used as tracers. Six liters of one twelfth strength Hoagland's solution were prepared for each absorption period of the experiment, this dilute solution being used in order that the absorption might be largely against a concentration gradient. For the phosphate translocation experiments, about one and one half microcuries of P^{32} per liter were added to the solution in which the phosphate concentration was 0.000083 M. In the rubidium translocation experiments, the nutrient solutions were made equimolar (0.005 M) with respect to rubidium and potassium by the adition of RbCl. About two microcuries of Rb^{86} per liter were used.

Analyses were made for radioactivity and total sugars. Harvested plants were cut into root and shoot, roots were blotted, and fresh weights taken. The roots were killed and thrice extracted in boiling 80% ethanol. The alcohol was evaporated from the combined extracts on a warming plate, the residue made to volume, and aliquots analyzed for radioactivity. Total sugars were estimated as glucose from the remaining extract by the method of Forsee (**9**) with one modification; the extract was treated with invertase prior to clearing and decolorizing. It was found in testing the suitability of the method that if this was not done there was a loss of sucrose during the decolorizing step. Iodine tests on the extracted roots always failed to show the presence of starch. The root residue remaining after extraction was also analyzed for radioactivity in the experiments where P^{32} was used, for about one third of the total activity could not be extracted. In experiments using Rb^{86}, the extractions left so little radioactivity in the roots that it was scarcely discernible.

The dried shoots were wet-ashed in nitric acid and made to volume with dilute hydrochloric acid. Analysis for radioactivity was made with a Geiger-Muller counter on 2-ml. aliquots of solution, or extract, evaporated to dryness in porcelain capsules. All counting was at constant geometry. Conversion factors for expressing activities as micromoles of ion were obtained by determining the radioactivity of the nutrient solutions prepared for the experiments.

The amount of tracer ion found in the shoot at the termination of the experimental period represents that quantity of ion translocated across the

root, released to the xylem, and carried upward in the transpiration stream. It is not known how the tracer ion retained in the root is distributed. Portions of the total amount of ion must be resident in the protoplasm as part of the living system, or as ions in transit to the vacuoles and xylem; portions must be present in the cell sap, and there should be small amounts in the root xylem which have not yet been translocated upward to the shoot.

Experimental results

Time course of rubidium translocation to the shoot during day and night

This experiment was designed to determine how much time would elapse before detectable amounts of rubidium could be transported from the nutrient medium to the shoots. Low salt plants were cultured as it was believed that minimal translocation to the shoots would occur with this status. The solutions prepared for this experiment contained but 0.000075 molar RbCl. Starting at 10:00 A.M. on the day of the experiment, 12 plants were transferred to pans of the radioactive nutrient solution, replaced under the lights, and vigorously aerated. At one-, two-, and four-hour intervals, four individuals were harvested. At 2:00 A.M. of the following night the same procedure was carried out for 12 more plants. These latter plants were exposed

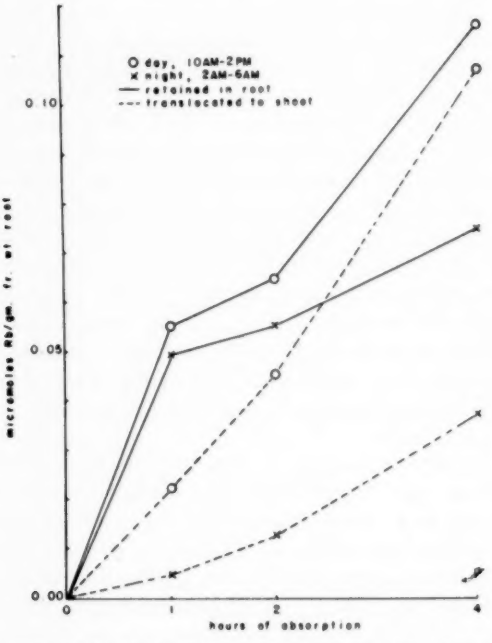

Fig. 1. Amounts of absorbed rubidium translocated to the shoots and retained in the roots of low salt bean plants during night and day absorption from dilute RbCl.

to indirect light from an incandescent bulb for a few minutes at the beginning of the experiment and again during each harvest.

The pertinent results of the experiment are depicted in figure 1. Each value represents the mean of four plants. Data on both the retention and translocation of rubidium are expressed per gram fresh weight of root. It is considered that since the root must perform the work essential to translocation of the ion to the xylem, such an expression is preferable to one based on the weight of the shoot.

The amount of rubidium retained by the roots in the first hour is much alike for both day and night absorption, and probably results from similar adsorption on the root surface by ion exchange. The rate at which rubidium is gained by the root after the first hour must more nearly reflect the capacity of the root to retain absorbed rubidium in cell sap or protoplasm; obviously, roots absorbing during the day had the greater capacity to accumulate rubidium. The most striking difference between night and day behavior occurred in the amounts of rubidium translocated to the shoot. Translocation was far greater during the day, whether expressed in absolute amounts or in per cent. of total ion absorbed and even during the first hour of absorption when the amounts of rubidium retained by the roots were similar. At the end of four hours nearly half of the rubidium absorbed during the day was in the shoot; at night, only one third had been translocated upwards. It was decided that in subsequent work a four-hour absorption period would be used in order to make certain that the amount of rubidium found in the root would reflect something of the metabolic status of the root, and not just ion exchange.

Diurnal variation in the amount of absorbed rubidium translocated to the shoot

This experiment was designed to provide information on the diurnal variation in the amounts of rubidium absorbed, and on the relative distribution of the ion between root and shoot. Forty-eight plants were raised, 24 each of the low and high salt status. The experimental day was divided into six consecutive four-hour absorption periods, beginning at 6:00 P.M. when the lights were turned off. Six pans of the dilute Hoagland's solution plus RbCl and Rb^{86} were prepared, one for each absorption period. At the beginning of each period four low salt and four high salt plants were placed in a pan of the radioactive solution and replaced under the light canopy. The experimental solution was vigorously aerated to effect movement of the solution about the roots. At the end of four hours the plants were harvested.

Figure 2 presents the results of the experiment. It is evident that the plants were more active in absorbing, retaining and translocating rubidium during the day. Minimum root activity occurred about midnight, maximum about midday. As was indicated in the initial experiments, the ability of the root to translocate rubidium to the xylem was increased by the coming of day to a greater extent than was its ability to retain the ion.

The percentage data show that there was a significant increase in the

ability of the root cytoplasm to translocate rubidium from about midnight to early morning, but the greatest increase occurred with the coming of day. Low salt plants showed this increased translocation to a greater degree than did high salt plants, which is not in accord with the view that low salt plants tend to hold more of the absorbed ion in the root. Due to the acceleration of translocation with the coming of day, there was no consistent relationship between the percentage translocated and the total amount of rubidium absorbed.

No consistent correlation can be found between the total sugar content of the root and absorption, translocation, or retention. During the day some correlations might have appeared to exist, but they did not hold at night. If total sugar content of the root controlled the activity of the root, one

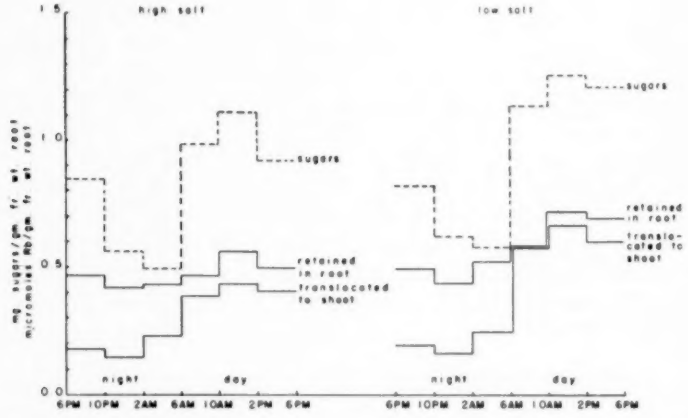

FIG. 2. Diurnal variation in the amounts of rubidium retained in the roots and translocated to the shoots of high and low salt bean plants during four-hour absorption periods.

would look for the closest correlation to occur at night when sugars might become limiting. As it is, the roots began increasing in activity during the last period of the night when the sugar content of the root was at a minimum.

The sugar content of the roots was quite low, which probably reflects their culture under low light intensities with favorable temperatures and oxygen supply. The fact that the low salt roots had but slightly higher sugar content than the high salt roots suggests that these plants did not represent the extremes of salt status usually associated with the terms. However, the capacities of the two types of plants to absorb rubidium were clearly different, and the difference in the per cent. of absorbed ion translocated during the day must also have resulted from the difference in salt-sugar status.

In general, the results are in agreement with the findings from exudation studies; that is, there exists a diurnal fluctuation in the activity of the root which is here reflected in rubidium absorption and translocation.

Diurnal variation in the amount of absorbed phosphate translocated to the shoot

This experiment was designed to provide the same information on the translocation of phosphate as was obtained for rubidium. It was performed in the same manner, except that no RbCl was present in the dilute Hoagland's solution used in the experimental periods. Figure 3 gives the results of the experiment. There were some similarities between the results for phosphate and rubidium. The time course of translocation to the shoot was

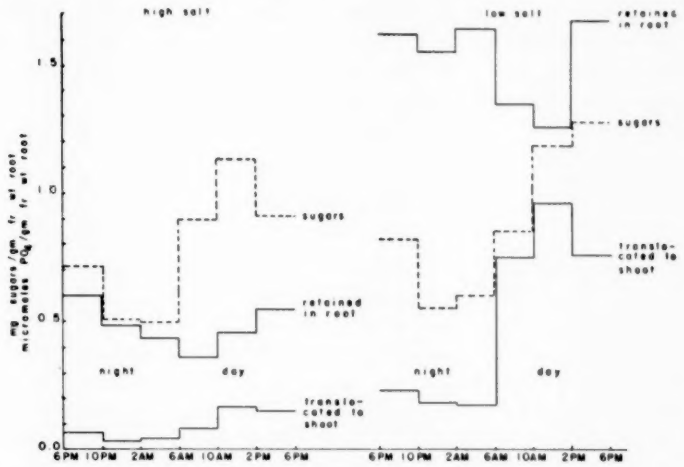

Fig. 3. Diurnal variation in the amounts of phosphate retained in the roots and translocated to the shoots of high and low salt bean plants during four-hour absorption periods.

of the same nature for both ions, with the minimum reached about midnight and the maximum about midday. Again, there was no consistent correlation between total sugar content and root activity in absorption, retention, or translocation. Unlike rubidium, however, phosphate was not retained in the roots in greater quantity during the day; rather, there was a significant decrease in phosphate retention. The data for high salt plants, in which there was less variability between the plants of any one period, clearly showed that the diurnal cycles for retention and translocation were out of phase, with the low point in retention being reached during the first period of the day. The corresponding cycles for rubidium were in phase, though differing in amplitude. In general, it appears that the increased daytime absorption of phosphate by bean plants was largely accounted for by the increased transfer of the ion to the shoot; whereas the increased daytime absorption of rubidium was due to increased capacity of the root to retain the ion as well as to increase transference to the shoot.

Although the percentages of absorbed ion translocated to the shoot were much higher during the day for both phosphate and rubidium, there were

striking differences shown for the two ions (fig. 4). A smaller percentage of phosphate was transferred to the shoot than was the case with rubidium. The increase in percentage of phosphate translocated was delayed until the first period of the day, while with rubidium there was a significant increase during the last period of the night. There was a significant decrease in the percentage of phosphate translocated during the last period of the day, while this was not true for rubidium. The diurnal change favoring rubidium transport across the root symplast began earlier and continued longer than was the case with phosphate transport.

Percentagewise, as well as in absolute amounts, low salt plants translocated more phosphate and rubidium to the shoot in a four-hour period than did high salt plants, but the salt status had a much greater effect in the case of phosphate. In general, the increased activity of the root brought about by the low salt status was much more apparent with phosphate than with rubidium.

The effect of darkness during the normal day period on the translocation of phosphate to the shoot

If, as suggested by the previous experiments, there is a change in the bean root symplast with the coming of day which tends to increase the proportion of absorbed ion passed into the xylem, there should be detectable increases in translocation during the day period even when the plants are kept in darkness. This experiment was designed to test this postulate. As low salt plants showed a tremendous surge in translocation of phosphate to the shoot in the first period of the day, it was decided to use this salt status and ion in order to gain the greatest sensitivity possible.

Procedures were those of the preceding experiment. The additional dark

TABLE I

TRANSLOCATION OF ABSORBED PHOSPHATE TO THE SHOOTS OF LOW SALT BEAN PLANTS UNDER DAY CONDITIONS OF LIGHT AND DARKNESS.

Light condition	Period	Phosphate			Phosphate translocated to shoot	Sugar in root
		Retained in root	Translocated to shoot	Total absorbed		
		micromoles/gm. fresh wt. root			%	*mg./gm. fresh wt.*
Dark	6 P.M.–10 P.M.	1.056	0.081	1.137	7.2	0.90
	10 P.M.– 2 A.M.	1.174	.079	1.253	6.2	.72
	2 A.M.– 6 A.M.	1.179	.083	1.261	6.6	.56
Light	6 A.M.–10 A.M.	1.072	.486	1.558	31.3	1.02
	10 A.M.– 2 P.M.	1.200	.715	1.915	37.4	1.42
	2 P.M.– 6 P.M.	1.221	.470	1.690	27.2	1.41
Dark	6 A.M.–10 A.M.	1.011	.166	1.177	14.0	.30
	10 A.M.– 2 P.M.	1.070	.172	1.242	13.7	.34
	2 P.M.– 6 P.M.	.987	.095	1.082	8.9	.31
L.S.D. 5%*		0.262	0.126	0.404	3.3	0.16

*Least significant difference at the 5% confidence level.

treatment during the normal day period was given by placing 12 plants in a dark chamber constructed in the growth room. Results are given in table I. There was a great deal of variability in the individual plants of this experiment, as was always the case with low salt plants, and there are no significant changes in the amounts of absorbed phosphate retained in the roots; only the difference in phosphate retained by the roots of darkened and illuminated plants during the 2:00 P.M. to 6:00 P.M. period approached statistical significance. With phosphate translocation to the shoot, however, the response to light was large and unmistakable, as it was in the previous experiment. Those plants that were kept in the dark during the day period

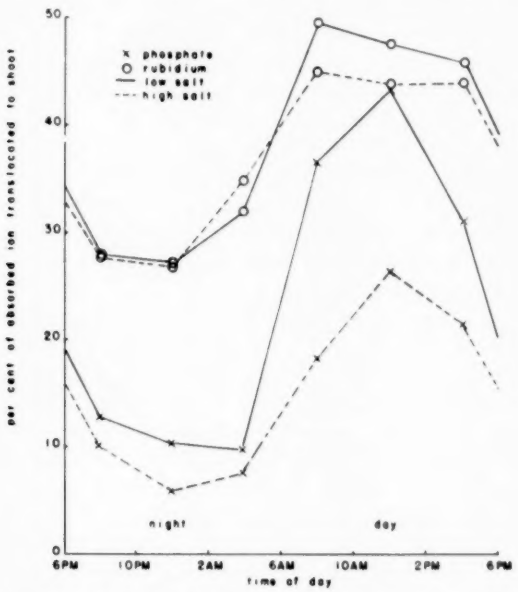

FIG. 4. Diurnal variation in the percentages of rubidium and phosphate absorbed in four-hour periods that are translocated to the shoots of high and low salt bean plants.

also showed an increase in translocation over the night level, but it missed significance. However, the per cent. of absorbed phosphate which was translocated to the shoot showed that the diurnal change in favor of transport across the symplast occurred with the normal coming of day even in darkened plants. Darkened plants absorbed about the same amount of phosphate during the day as they had during the previous night, but the proportion transported to the shoot was much larger.

It seems, then, that there exists a diurnal variation in the ability of the bean root symplast to transport phosphate to the xylem, but it is apparent that illumination of the plant serves to greatly augment this basic change. Again, most of the increased daytime absorption of phosphate is accounted for in the increased translocation of the ion to the shoot.

The effect of added sucrose on the translocation of absorbed ions by darkened plants

This experiment was performed to ascertain if adding sucrose to the nutrient medium would increase the rate of translocation of phosphate and rubidium to the shoots of darkened bean plants. Although there is some evidence that root-supplied sucrose is ineffective in increasing exudation (**19**), and it is stated that the influence of sugars on ion secretion into the xylem appears to be weak (**2**), incomplete experimentation in this laboratory shows that under some conditions (dilute sucrose in Hoagland's solution applied in the morning to roots of bean plants that have not been in the dark longer than overnight) applied sucrose will definitely increase the exudation of rubidium.

Forty-eight plants were raised for this experiment and were used in lots of 16. The first lot was used to test the effects of light and sucrose in maintaining the translocation rate of phosphate during the transition from day to night. Four plants were used in the absorption period from 2:00 P.M. to 6:00 P.M. to establish a basic rate for retention and translocation from which to measure deviations in the subsequent night period. The remaining plants were divided into three treatments for the 6:00 P.M. to 10:00 P.M. period: dark (control), continued light, and dark plus 0.005 molar sucrose in the experimental nutrient solution.

The two remaining lots of plants were used the following morning in a study of the effect of continued darkness and darkness plus sucrose on the acceleration of ion translocation that occurs as the plant passes from night to day. Phosphate was traced in one lot, rubidium in the other. Four plants from each lot were used in the 2:00 A.M. to 6:00 A.M. absorption period, again to establish a base from which to measure changes in the subsequent day period. There were three treatments in the 6:00 A.M. to 10:00 A.M. period: light (control), dark, and dark plus 0.005 molar sucrose in the nutrient solution.

Solutions and procedures were as previously described. The darkened plants were again held in the dark chamber in the growth room. The results of the experiment are displayed in table II. The evening experiment indicated that the phosphate retained by the root significantly increased if the plants were exposed to continued light in what is normally the first period of night. In darkness during the same period, there was no significant change in the amount of phosphate retained and the addition of sugar to the nutrient medium had no significant effect. Translocation of phosphate to the shoot was maintained in the day-to-night transition if the light continued, but fell if normal darkness followed the day. The percentage figures show the usual lower per cent. of translocation in the night under all treatments.

In the morning experiment the only significant change in phosphate retention with the passage from night to day was in the loss shown by plants kept in continued darkness. Light produced a large increase in translocation, but no significant change in retention. Applied sucrose increased the

retention of phosphate in the roots of darkened plants without producing any change in the amount of translocation. It is possible that the increased retention of phosphate in the root due to applied sucrose was not related to the problem studied here, but that it reflected the phosphorylation process associated with sucrose absorption (**16, 18**).

The data on percentage of absorbed phosphate translocated to the shoot again indicate that some diurnal change favoring transport across the root occurs with the normal coming of day. Some factor or factors associated with light accentuate this change. A comparison of the morning and evening

TABLE II

THE EFFECT OF SUCROSE APPLIED TO THE ROOTS OF DARKENED BEAN PLANTS ON THE DISTRIBUTION OF PHOSPHATE AND RUBIDIUM BETWEEN ROOT AND SHOOT.

Traced ion	Period	Light condition	Ion			Ion translocated to shoot
			Retained in root	Translocated to shoot	Total absorbed	
			micromoles/gm. fresh wt. root			%
PO_4	2 P.M.– 6 P.M.	Light	1.005	0.073	1.078	6.7
	6 P.M.–10 P.M.	Dark	1.143	.020	1.163	1.8
	6 P.M.–10 P.M.	Light	1.716	.063	1.779	3.4
	6 P.M.–10 P.M.	Dark plus sucrose	1.283	.018	1.301	1.4
L.S.D. 5%*			.588	.035	.619	1.0
PO_4	6 A.M.– 6 A.M.	Dark	1.229	.033	1.332	2.5
	6 A.M.–10 A.M.	Light	1.338	.132	1.470	8.9
	6 A.M.–10 A.M.	Dark	.932	.070	1.003	6.8
	6 A.M.–10 A.M.	Dark plus sucrose	1.482	.074	1.556	4.8
L.S.D. 5%*			.378	.040	.384	2.7
Rb	2 A.M.– 6 A.M.	Dark	.553	.197	.750	26.2
	6 A.M.–10 A.M.	Light	.654	.402	1.056	38.1
	6 A.M.–10 A.M.	Dark	.586	.222	.808	27.5
	6 A.M.–10 A.M.	Dark plus sucrose	.604	.246	.850	29.0
L.S.D. 5%*			0.040	0.030	0.062	1.6

*Least significant difference at the 5% confidence level.

transitions shows that light increases phosphate translocation in both cases but that the diurnal cycle is not to be denied. In the evening, continued light operates in opposition to the diurnal change; in the morning light operates with it. Increased sugar content may be the principal factor increasing translocation provided that it is supplied through the phloem, but when applied through the nutrient solution it is without effect.

The behavior of the plants with respect to rubidium was somewhat different than with phosphate. Some of these differences have been previously noted; a much greater percentage of rubidium was translocated to the shoot under all conditions, and the passage from night to day was accompanied

by large increases in both translocation and retention of the ion. Again, rubidium translocation was not accelerated by the coming of day to the same degree as was the case with phosphate. The change produced by applied sucrose was in the same direction as that produced by light, and since a linear relationship seems to exist between rubidium absorbed and rubidium translocated in the 6:00 A.M. to 10:00 A.M. period, it is unnecessary to postulate light factors other than photosynthesis to account for the acceleration of translocation induced by light. It should be noted that these low salt plants have translocated a much smaller percentage of phosphate than was the case in previous experiments. The reason for this is not known.

Discussion

This study shows that the probability that an ion entering the root symplast will be transported to the xylem depends on at least the following factors: (a) the time of day, (b) the species of ion concerned, (c) the salt status of the plant, and (d) light.

The reasons for a diurnal fluctuation in root activity are no more obvious in these experiments than they are in exudation studies to which one would expect them to be related if exudation is largely an osmotic phenomenon (**4**). For the present it must suffice to recognize that the root symplast possesses some inherent capacity to pass greater proportions of absorbed ion into the xylem during the day. This change is particularly true in the case of phosphate. With rubidium, the coming of day brings increases in both the amount of ion retained in the root and in the amounts translocated to the shoot, but the latter shows the greater increase. It is curious, too, that the change favoring rubidium translocation should begin before the coming of day and last throughout the light period, while increases in per cent. of phosphate translocated begin with the day and fall off in the afternoon. There is no obvious explanation for this difference in behavior.

The results obtained here are not in accord with the findings of Hoagland (**10**) and Broyer (**5**) that higher salt plants pass proportionately more ion to the xylem during the initial stages of absorption. It was in the low salt plants that the greatest percentage of absorbed ion was translocated to the shoot, particularly with phosphate during the day. It is conceivable that in the first hour or two of absorption the high salt bean roots might have passed proportionately more phosphate and rubidium to the xylem, but if this were the case the low salt roots must have had a very great acceleration of transport across the symplast in the latter portion of the four-hour absorption period in order to end the period with a greater amount translocated. It seems more likely that the low salt roots were more active in ion translocation from the beginning of the absorption period. Since Hoagland and Broyer did not actually compare plants cultured with different amounts of available salt, but rather low salt plants that had, and had not, been allowed to accumulate some salt in a previous absorption period, their experiments are not strictly comparable with these.

It is difficult to say with certainty that the effect of light on increasing ion translocation to the xylem is due solely to an increased supply of metabolite to the root. These experiments, however, suggest that increasing the root activity by increasing the root sugar content via the phloem serves to increase the percentage of absorbed ion translocated to the shoot. It is possible that light might have other entirely different effects tending to increase translocation, but this study is not adequate to disclose them.

Summary

A diurnal variation has been found to exist in the ability of Red Mexican bean roots to translocate absorbed rubidium and phosphate to the shoots. The maximum translocation occurs about midday, the minimum about midnight. The source of variation appears to reside in some obscure property of the root cytoplasm.

Superimposed upon the diurnal variation are effects due to the metabolic status of the roots. Within any one period of the day, those roots that absorb the most ion by reason of an adequate supply of metabolite, a low-salt, high-sugar nutritional status, or possibly some unknown factor associated with illumination, will also translocate a greater percentage of the ion to the shoot.

Rubidium and phosphate demonstrate quite different behavior in the bean plant. Both ions are absorbed in greater quantity during the day as compared to night, but the increase in phosphate absorption is largely accounted for by increased translocation to the shoot, while the increase in rubidium absorption is due to both increased translocation and accumulation within the root. Greater percentages of rubidium are translocated to the shoot than is the case with phosphate, and the daily increase in percentage of ion translocated begins earlier and continues longer with rubidium. The low salt nutritional status produces a much greater increase in the absorption and translocation of phosphate than it does of rubidium.

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HEAVY-METAL NUTRITION AND IRON CHLOROSIS OF CITRUS SEEDLINGS

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The recent widespread increase in iron chlorosis on acid, sandy soils in Florida has led to studies of the underlying causes. The leaves on affected trees are deficient in iron (**10**). Field observations show that tree condition can be substantially improved by liming. Additions of iron sulphate to the soil has not improved tree condition noticeably. Recently foliage condition improved when small quantities of chelated iron were used (**12**). It is not known yet whether soil applications of this material will promote the rehabilitation of the topsoil fibrous root systems, which have been severely damaged in all cases investigated. The heavy metals Cu, Zn, and Mn accumulate in the topsoil of citrus orchards despite its very sandy and acidic nature (**8**). This accumulation is independent of that of phosphate, which takes place simultaneously (**8**). These metals, as well as others, may interfere with Fe metabolism of plants (**3, 5**).

Little is known about the fundamentals of heavy-metal nutrition of citrus. There is actually little information on the relative toxicities, antagonistic reactions, or the nature and extent of the accumulation of heavy metals in roots of any plant. Much additional research is needed to elucidate the processes of their absorption and accumulation. The present studies were made with the hope that some information on these aspects of nutrition could be gained and it is felt that the results presented will contribute toward this end.

Materials and methods

The plants tested were young citrus seedlings grown in seedbeds of vermiculite as described elsewhere (**9**). Valencia orange seedlings were used mostly; however, some Sunki mandarin seedlings were included in one experiment. The seedlings were one to two months old when used and the experiments were continued two or three months, which gave sufficient time for a widely differential growth response.

Two systems of culture were used. The first involved use of clay pots filled with vermiculite and placed in glazed pottery vessels which permitted sub-irrigation with the desired nutrient solution. The solution was flushed through the pot twice weekly and adjusted frequently to keep the pH near 4 and to maintain the liquid level. Fresh solutions were applied about every two weeks. The second system was a solution culture in one-quart glass jars that had been painted successively on the outside with asphalt and

aluminum paints. The solutions were adjusted to pH 4 and brought to volume with distilled water weekly and new solutions were supplied every four weeks. In addition, the solutions were tested every few days to see that the applied levels of Cu, Zn, Mn, and Fe remained nearly constant. In both systems of culture two seedlings were grown in each container.

The first vermiculite experiment had two sources of nitrogen (all NO_3 and half NH_4), two levels of Cu (0.02 and 10 p.p.m.), two levels of Zn (0.05 and 15 p.p.m.), and two levels of Mn (0.5 and 25 p.p.m.). All possible combinations of these variables were used, making a total of 16 solution treatments. Iron from ferric tartrate was added thrice weekly at the rate of 0.5 p.p.m. Fe. Nitrogen and the other unmentioned essential elements were supplied here and elsewhere in levels equivalent to those in one fourth strength Hoagland's culture solution. After 90 days these plants were harvested, divided into roots, stems, and leaves, rinsed several times in distilled water, dried and analyzed spectrographically for 10 elements. Only the heavy metal analyses are reported. Zinc was determined by standard chemical procedures. The second vermiculite experiment and the solution cultures had 75% of the N supplied as NO_3. The ammonium ion was used to retard shifts in pH. Four levels of Cu (0.02, 2.5, 5, and 10 p.p.m.), three Zn levels (0.05, 25, and 75 p.p.m.), and three Mn levels (0.5, 25, and 75 p.p.m.) were used in combination with two Ca levels (40 and 160 p.p.m.) and two Fe levels (1 and 5 p.p.m. from ferric tartrate). This made a total of 40 cultures, supplemented by a few additional 10 p.p.m. Cu cultures which contained 10 p.p.m. Fe from the chelated complex, KFe-EDTA (6). This experiment was also continued for 90 days but the only tissue analysis made was for Fe in chlorotic leaves.

The solution culture experiment had variable quantities of Cu, Zn, and Mn each at two levels of chelated Fe. These treatments are shown in detail in table IV. Each treatment was made in triplicate. The plants were grown under differential treatment for 70 days. Only visual observations of the growth responses were made.

Differential quantities of the heavy metals were supplied by using appropriate quantities of $CuSO_4$, $ZnSO_4$, $MnCl_2$ and ferric tartrate or ferric iron from the chelated complex mentioned previously.

Results and discussion

Toxicity of heavy metals and their effects on growth

In the vermiculite cultures, growth was very good except in the supplementary Cu and 75 p.p.m. Zn treatments, which all caused chlorosis or stunting (fig. 1, C to G). Copper was the most toxic of the three heavy metals, causing severe suppression of root growth and inducing chlorosis at much lower levels of supply than either Zn or Mn. Neither the source of N nor the Ca level had any visible effect on the behavior of the heavy metals. In the first vermiculite experiment where various combinations of the metals were added, there did not appear to be any additive effect on growth suppression by the three elements. Solutions containing 10 p.p.m. Cu caused

severe stunting and chlorosis of the very few leaves that developed regardless of the amounts of Zn and Mn. Zinc at the rates of 15 and 25 p.p.m. and Mn at all rates including the 75 p.p.m. permitted normal growth of both roots and tops. Examples of plants grown at certain levels of the metals are shown in figure 1, A to F.

The frequent additions of ferric tartrate did not prevent the development of chlorotic leaves in the supplemental Cu cultures of the first test. In the

FIG. 1. Effect of certain heavy-metal treatments on growth and chlorosis of citrus seedlings in vermiculite and solution cultures. All are Valencia orange except the plant on the right in each clay pot which is a Sunki mandarin seedling. The full-nutrient solution contained the following supplemental metals in p.p.m.: A, none; B, 75 Mn; C, 75 Zn; D, 2.5 Cu; E, 5 Cu; F, 10 Cu; G, 10 Cu; H, none; I, 0.25 Cu; J, 3 Zn; and K, 5 Mn. Solutions for D, E, and F contained 5 Fe from ferric tartrate. Solution for G contained 10 Fe from KFe-EDTA. Ruler is six inches long.

second vermiculite experiment, where Fe was a variable, no difference in growth, intensity of chlorosis, or reduction in metal toxicity to roots was associated with the rate of Fe-tartrate supplied. The use of chelated Fe, however, prevented the development of chlorosis but did not overcome the suppressive effect of Cu on growth. (Compare A, F, and G, fig. 1.)

Living roots that contained 500 or more p.p.m. Cu by dry weight were found to be sharply altered in appearance. Elongation was interfered with so that numerous laterals were arrested in growth and looked like pegs along their parent root. These roots were usually swollen and had a cavity where the apical meristem should normally be. Their color varied from very light to dark brown. This apparently depended on the amount of Fe supplied and the ratio Mn : Fe. A high Mn : Fe ratio in the solution gave very light-colored roots. The toxic effect of Cu on the roots is much like that described for Zn (**3**).

Vermiculite has a relatively high exchange capacity (**1**). It was found near the end of these experiments that nearly all of these heavy metals were adsorbed by the vermiculite even though the pH was maintained near 4. This apparently reduced greatly the effectiveness of these elements as much lower concentrations in the solution cultures showed marked effects on growth. It seems probable that the results obtained in vermiculite would be somewhat comparable to those that would be found under acid soil conditions. Chlorotic seedlings with stunted roots are of rather common occurrence under citrus trees in Florida orchards when Cu is high and pH low.

The uniformity of response in the nutrient solution experiment was good. The plants in favorable solutions showed growth that was equal or superior to that in good soil or in vermiculite with applied nutrient solutions. Root development was surprisingly good (fig. 2) despite the lack of mechanical aeration. Under these conditions similar stunting and chlorosis were caused by all three heavy metals (fig. 1, H to K). This picture and those in figure 2 were taken 70 days after treatments were begun.

The observations reported in table I were made on the sixty-first day. The suppression of growth as Cu was increased is evident. The greatest intensity of chlorosis occurred at the intermediate Cu levels rather than at the highest because some growth must occur before chlorosis can appear. One p.p.m. Cu was very toxic and lethal to some roots, with the consequence that new leaf development was essentially curtailed. When the chelated Fe was increased from 0.5 to 5 p.p.m. the Fe chlorosis pattern in the new leaves never developed but after several weeks they showed the distinctive Mn deficiency pattern (**2**). The increased Fe was slightly toxic at the lowest levels of the other metals, showing some leaf scorch and some reduction in new leaf production (table I). The scorch was identical with that produced by ferrous sulphate (**2**). No beneficial effect of the increased Fe on root growth was noted at any Cu level used. The absence of Fe chlorosis in the leaves made the high-Fe plants appear better for the first few weeks, but their general appearance after two months showed only doubtful improvement. About the only difference was the replacement of Fe chlorosis with

Mn chlorosis in the leaves as a symptomatic expression of an extremely unhealthy root condition.

The word "stunted" as used in table I indicates severe suppression of root growth and many pegged laterals. None of the roots were dead. The term

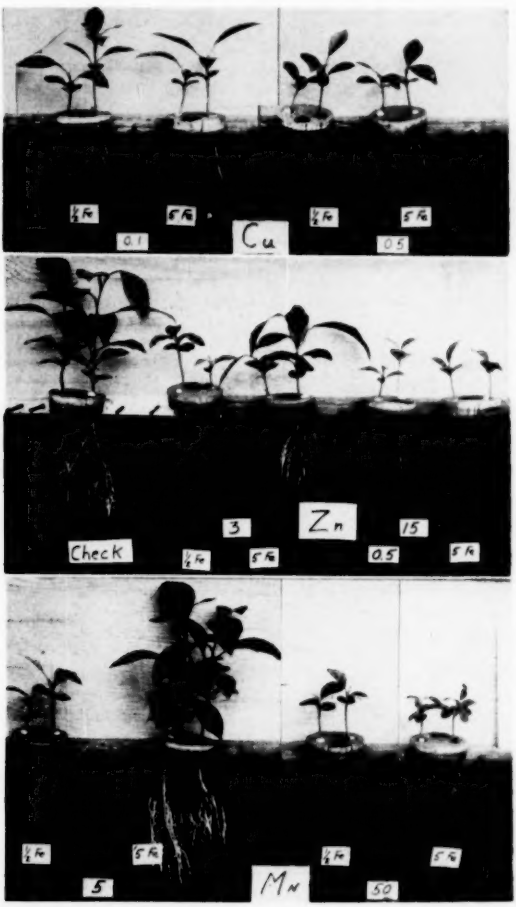

FIG. 2. Valencia seedlings grown in solution cultures with two levels each of Cu (0.1 and 0.5 p.p.m.), Zn (3 and 15 p.p.m.), Mn (5 and 50 p.p.m.), and Fe (0.5 and 5 p.p.m.). The plants with 0.5 Cu and 5 Fe do not show chlorosis but the roots are severely stunted (compare with check in center row).

very stunted is used to indicate extreme metal toxicity and presence of some swollen (bulbous) root tips. The root systems are capable of new growth when placed in a favorable environment. The roots indicated as dead were flaccid, discolored, and in various stages of decomposition. In appearance the plants at 3 p.p.m. Zn were about like those at 0.25 p.p.m. Cu. Actually

the root condition was somewhat better. The 15 p.p.m. Zn plants were likewise similar to the 1 p.p.m. Cu plants. Thus, under these conditions Cu seems to be about 12 to 15 times as toxic as Zn. Both elements block the upward translocation of Fe and thus induce Fe chlorosis in the leaves. The increased level of chelated Fe eliminated this symptom with Zn also and this

TABLE I

GROWTH RESPONSES OF VALENCIA ORANGE SEEDLINGS AFTER TWO MONTHS IN COMPLETE NUTRIENT SOLUTION WITH HEAVY METALS VARIED AS INDICATED.

Cu	Zn	Mn	Fe	New leaves*	Chlorosis type	General appearance of tops	Condition of roots
p.p.m.	*p.p.m.*	*p.p.m.*	*p.p.m.*				
0.004	0.01	0.1	0.5	32	....	Very good	Very good
0.1	0.01	0.1	0.5	19	Fe	Fair	Stunted
0.25	0.01	0.1	0.5	13	Fe	Poor	Very stunted
0.5	0.01	0.1	0.5	8	2 Mn 3 Fe**	Poor	Very stunted; partly dead
0.75	0.01	0.1	0.5	6	Fe	Poor	Very stunted; partly dead
1	0.01	0.1	0.5	4	Fe	Poor	Very stunted; mostly dead
0.004	0.01	0.1	5	25	Mn	Good; slight Fe toxicity	Good
0.1	0.01	0.1	5	22	Mn	Fair	Stunted
0.5	0.01	0.1	5	12	Mn	Poor	Very stunted; partly dead
1	0.01	0.1	5	9	Mn	Poor	Very stunted; mostly dead
0.004	3	0.1	0.5	12	Fe	Poor	Slightly stunted
0.004	15	0.1	0.5	4	Fe	Very poor	Very stunted; partly dead
0.004	30	0.1	0.5	0	....	Five dead	All dead
0.004	3	0.1	5	19	Mn	Fair	Slightly stunted
0.004	15	0.1	5	2	Mn	Very poor	Very stunted; partly dead
0.004	30	0.1	5	0	....	All dead	All dead
0.004	0.01	5	0.5	16	Fe	Fair	Slightly stunted
0.004	0.01	25	0.5	6	Fe	Poor	Stunted
0.004	0.01	50	0.5	3	Fe	Poor; Mn toxicity	Stunted; partly dead
0.004	0.01	5	5	37	....	Very good	Very good
0.004	0.01	25	5	26	....	Good	Slightly stunted
0.004	0.01	50	5	22	....	Poor; Mn toxicity	Stunted
0.004	0.01	0.1	25	0	....	Dead	Died after tops

*Total on six plants.
**Of six plants, 2 showed Mn chlorosis, 3 Fe chlorosis.

again resulted in Mn-deficient leaves. The increased iron level in this case substantially improved the top growth but did not entirely overcome the toxic effect of Zn on the roots (fig. 2).

Manganese was much less toxic than Cu or Zn but readily caused Fe chlorosis. It would appear from these comparisons that Cu is about 50 times as toxic as Mn. At the low Fe rate, 5 p.p.m. Mn caused very severe

chlorosis and stunting of the top and the roots showed evidence of toxicity. Substantial improvement in both root and top condition was achieved by increasing the chelated Fe to 5 p.p.m. (fig. 2). Plants with 5 p.p.m. Mn and 5 p.p.m. Fe were among the best in the experiment. At 50 p.p.m. Mn caused severe chlorosis and stunting of the tops and was toxic to both leaves and roots. With Fe increased to 5 p.p.m. chlorosis of the leaves was prevented but yellow orange spots and curling caused by Mn toxicity (**4**) developed. The roots in these cultures also showed typical heavy metal toxicity. Even 25 p.p.m. Fe appeared to be non-toxic to roots. Excessive absorption of chelated Fe, however, killed the leaves in a few days. The initial symptom of Fe toxicity was a speckled scorch, followed by death of the leaf. The roots were still alive and light-colored several days after the leaves and stems were dry and brown. Cu, Zn, and Mn at the supplementary levels shown in table I, each prevented the leaf scorch that 5 p.p.m. Fe caused at the low levels of these elements. A few supplementary jars were given 25 p.p.m. chelated Fe along with the high rates of Cu, Zn, and Mn; *i.e.*, 1, 30, and 50 p.p.m. respectively. No Fe leaf scorch appeared in any of these combinations but the roots still showed evidence of heavy metal toxicity. The tops were not chlorotic in any of the treatments but they were severely stunted.

The relative effects of Cu, Zn, and Mn on citrus seedlings are of the same order as described by Hewitt (**5**) for sugar beets. His observations were apparently limited to the upper parts of the plant since root behavior was not mentioned. He states that it is possible to induce chlorosis with these and other metals at levels that are non-toxic to plants. All the present observations on Cu and Zn on citrus have shown that toxicity to roots either accompanied or preceded leaf chlorosis. The effect of Mn was not clearly established. It may be possible to find conditions under which Mn would induce Fe chlorosis without being toxic to roots. It appeared in the present tests, however, that Mn reduced root growth before chlorosis developed in the leaves. The responses of different plant species may vary somewhat, of course.

The development of Mn-deficiency symptoms after correction of Fe chlorosis in plants grown with excess Cu and Zn agrees with the published data (**10**) in which it was shown that both Fe and Mn were at very low levels in the leaves of chlorotic orchard trees. It would seem logical that this same response might occur in orchard trees that were treated with chelated iron. The failure of iron to reduce the toxicity of Cu to roots in these tests would seem to suggest that the application of chelated Fe to chlorotic citrus trees in the orchard may not promote the re-establishment of roots in the soil zone containing relatively large amounts of accumulated Cu. The Fe may, however, be beneficial for other reasons. Thus, for example, a tree with restored photosynthetic capacity may develop additional roots in a different soil horizon or it may recover much more rapidly when Fe is used in conjunction with lime. The fact that citrus is much more sensitive to Cu than to Zn and Mn, along with the evidence (**7, 8**) that more Cu than either Zn or Mn has accumulated in the soil of afflicted orchards,

TABLE II

EFFECT OF NITROGEN SOURCE AND SUPPLIED LEVEL OF COPPER, ZINC, AND MANGANESE ON MEAN DRY WEIGHT (GRAMS PER PLANT) AND ON CONCENTRATION (P.P.M. IN DRY MATTER) OF ESSENTIAL HEAVY METALS IN VARIOUS PARTS OF VALENCIA ORANGE SEEDLINGS.

Treatment	Mean dry weight			Cu			Zn			Mn			Fe		
	Leaves	Stems	Roots	Leaves	Stems	Roots	Leaves	Stems	Roots	Leaves	Stems	Roots	Leaves	Stems	Roots
All NO_3	1.63	0.88	1.44	15.1	16.1	416	142	180	3,894	389	173	6,138	92	47	854
Half NH_4	1.83	0.88	1.30	19.3	25.2	372	127	164	3,009	229	98	3,460	91	45	1,216
L.S.D.	N.S.	N.S.	N.S.	4.2	5.3	N.S.	N.S.	N.S.	602	150	71	2,025	N.S.	N.S.	380
0.02 p.p.m. Cu	2.39	1.15	1.70	18.2	19.0	176	147	192	4,342	339	151	6,391	109	46	1,077
10 p.p.m. Cu	1.05	0.60	0.95	16.0	22.4	612	122	152	2,561	280	121	3,207	74	46	993
L.S.D.	0.18	0.20	0.31	N.S.	N.S.	65	16	40	1,430	N.S.	N.S.	2,050	18	N.S.	N.S.
0.05 p.p.m. Zn	1.79	0.90	1.31	18.0	20.4	402	89	131	2,400	328	159	5,829	98	45	975
15 p.p.m. Zn	1.62	0.87	1.38	16.2	21.0	387	179	210	4,500	290	113	3,769	85	47	1,095
L.S.D.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	14	47	1,449	36	19	1,678	N.S.	N.S.	N.S.
0.5 p.p.m. Mn	1.80	0.92	1.41	18.1	19.1	401	125	151	4,080	57	38	1,062	99	49	1,067
25 p.p.m. Mn	1.66	0.84	1.28	16.1	22.2	388	144	192	2,820	559	234	8,536	84	43	1,003
L.S.D.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	18	N.S.	N.S.	24	33	508	N.S.	N.S.	N.S.

L.S.D.—Least significant difference between means at odds of 19 to 1.
N.S.—Difference between means is not significant at odds of 19 to 1.

suggests strongly that excessive use of Cu is the chief factor involved in producing the chlorotic condition in Florida citrus trees on acid soil.

Effects of N source and rate of application of heavy metals on the distribution of the latter in citrus seedlings

Although N source had no effect on growth, it did have some effect on the behavior of each of the metallic ions (table II). Copper mobility was slightly increased by NH_4 so that more of it got to the tops of the plants. The reverse was true of Mn, while Zn and Fe were not affected. More Fe accumulated in the roots in the presence of NH_4 than in its absence. In contrast, less Zn and Mn accumulated in the roots in the NH_4 series.

One striking response was the absence of any apparent relation of leaf Cu concentration to the supplied amount or that accumulated in the roots. Thus, while the difference in rate of supplied Cu was 500-fold, the corresponding difference in the root concentration was only about four-fold and there was no difference in the leaf concentration.

Zinc was found to increase in all plant parts when the supply was increased. The root concentrations found at the two Zn rates differed even less than with Cu. The leaves, however, show a substantial increase.

The Mn concentrations in all tissues at the two rates of supply differed much more than was the case with either Cu or Zn. The ratio of the leaf concentrations is even larger than that of the roots. It is perhaps noteworthy that with this element the supply differential is only a factor of 50 instead of several hundred as with the other two metals.

The information just given in regard to heavy-metal distribution would suggest that Cu can induce Fe chlorosis without itself being transported to the foliage in increased amounts. If either Zn or Mn were the offending metal, however, one would expect to find an abnormally high foliage concentration of these elements. It has been reported (**10**) that chlorotic orange leaves from Florida orchards on acidic soil have unusually low Fe and Mn contents while Cu is present in normal amounts. Unpublished data have since shown that Zn concentration in such leaves is in the low-to-normal range. Thus, evidence from the pattern of metal distribution within the plant corroborates other evidence such as soil analysis and toxicity to roots in showing that excessive use of Cu is the chief factor that has brought on the chlorotic condition in many Florida citrus orchards.

Table II also shows how one metal may influence another. High Cu caused a reduction of Zn in all plant parts. It repressed Mn accumulation in roots and Fe in leaves. The Fe value of 74 is a mean from samples which contained mixtures of chlorotic and green leaves. Chlorotic leaves analyzed by themselves contained 26 p.p.m. Fe. Similar low Fe values were found in chlorotic leaves from plants grown in solution cultures with excess Zn or excess Mn. None of the metals influenced root accumulation of Fe however. Earlier work (**3**) showed this same relation between the Fe content of the roots and the leaves of lemon seedlings grown in solution cultures with excess Zn.

TABLE III

EFFECT OF NITROGEN SOURCE AND COPPER AND ZINC LEVELS ON THE CONCENTRATION OF MANGANESE IN VALENCIA ORANGE ROOTS.

Supplied metals	Concentration of Mn	
	Nitrogen all NO_3	Nitrogen half NH_4
p.p.m.	*p.p.m.*	*p.p.m.*
0.5 Mn:		
0.02 Cu plus 0.05 Zn	2,500	960
0.02 Cu plus 15 Zn	1,000	970
10 Cu plus 0.05 Zn	1,200	370
10 Cu plus 15 Zn	1,200	480
25 Mn:		
0.02 Cu plus 0.05 Zn	16,000	13,000
0.02 Cu plus 15 Zn	12,000	4,700
10 Cu plus 0.05 Zn	8,600	4,000
10 Cu plus 15 Zn	6,600	3,200

Zinc appeared intermediate between Cu and Mn in its effect on tissue composition. At the low rate of Cu supply, high Zn depressed the Cu concentration in the roots. At the high-Cu level, Zn had no effect on Cu accumulation. This interaction resulted in a non-significant overall effect of Zn on Cu uptake (table II). High Zn depressed Mn concentration in all tissues but had no effect on Fe distribution in this test where it had no effect on growth.

Manganese appeared to have little effect on the other metals. The effects of N source and Cu and Zn levels on accumulation of Mn by roots is shown in detail in table III. The effect of N source on Mn accumulation is clearly shown, but the cause is not understood. The suppression of Mn by Zn is obvious at the high-Mn rate but it was not consistent at the low-Mn rate at which it appears that the Cu level may have had some influence. Copper interfered with Mn accumulation regardless of Mn or Zn level. The effects of Cu and Zn were supplementary in the high-Mn treatments.

TABLE IV

CONCENTRATION OF OTHER HEAVY METALS IN VALENCIA ORANGE ROOTS LISTED IN THE ORDER OF INCREASING COPPER CONCENTRATION.

Rate of applied metal			Concentration in dry matter				
Cu	Zn	Mn	Cu	Zn	Mn	Fe	Al
			p.p.m.	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*
Low	High	Low	100	9,100	1,000	1,145	1,025
Low	High	Low	140	6,500	970	950	1,100
Low	High	High	170	3,950	8,350	1,250	1,220
Low	Low	Low	195	3,200	1,730	785	830
Low	Low	High	240	2,580	14,500	1,120	1,175
High	Low	Low	510	2,100	785	1,150	930
High	Low	High	550	1,920	6,300	810	1,025
High	High	Low	665	3,230	840	1,100	1,200
High	Low	Low	740	2,100	750	910	760

That Cu and Zn may influence the adsorption of each other is seen in table IV. Root analyses of plants from individual treatments were selected for the ascending Cu-values shown. No distinction was made between the N sources. Some evidence of antagonism between different pairs of elements may be seen, the most obvious being the reciprocal relation between the concentrations of absorbed Cu and Zn. The relation is not stoichiometric, nor does it appear to be controlled directly by metabolic processes (**9**). It may be possible to elucidate these adsorption responses by research on non-living exchange systems.

The data in table IV suggest that one element can simultaneously suppress the adsorption of two other elements. This adsorption can be seen by comparing the Cu, Zn, and Mn values in lines 1, 4, and 6, which represent treatments at the low-Mn level. When the rate of Zn was increased, both Cu and Mn were depressed. Likewise, when Cu was increased, both Zn and Mn were depressed. Similar effects at the high-Mn level may be seen in lines 3, 5, and 7. Aluminum was not supplied in these solutions. The vermiculite apparently supplied that which was adsorbed. The parallel behavior of Al and Fe is perhaps noteworthy since it agrees with the close parallel found in leaf accumulation of these two elements by orchard trees (**11**).

It seems apparent that the heavy metals influence the action of one another which points to the desirability of considering them as a group in studying heavy-metal nutrition. Single-element studies could lead to erroneous conclusions regarding ion behavior.

Summary

Studies were made on the responses to differential supplies of copper, zinc, manganese, and iron shown by sweet orange and mandarin seedlings grown in vermiculite and solution cultures with the pH maintained near 4.

The first three metallic ions were all found to interfere with Fe metabolism in such a way as to induce chlorosis under certain conditions. Relatively low concentrations of these elements also were toxic to roots. These two effects appeared to be related in that it was not possible to induce chlorosis without simultaneously inducing toxicity to roots. They were produced in solution cultures with 0.1 p.p.m. Cu, 3 p.p.m. Zn or 5 p.p.m. Mn, the lowest supplementary levels used. Copper appeared to be about 50 times as toxic as Mn and 12 to 15 times as toxic as Zn.

These metals appeared to cause Fe chlorosis by interfering with the upward movement of Fe since they did not reduce the accumulation of Fe in roots. Strongly chelated Fe overcame this interference in some way and prevented the development of chlorosis. It did not, however, prevent Cu toxicity in roots. It appeared to reduce slightly Zn toxicity in roots and to alleviate greatly toxicity due to Mn.

Chelated Fe from ferric potassium ethylenediamine tetra-acetate was toxic to leaves when used at 5 p.p.m. Fe in solution culture if all the other metallic ions were relatively low in concentration. The foliage scorch pro-

duced was identical with that produced by $FeSO_4$. This toxicity was prevented by increasing the amounts of any of the other three metals.

The analytical data presented show that Cu accumulates in roots, even to the point of toxicity, without being transported to the upper parts of the plants in increased amounts. Both Zn and Mn show marked accumulation in the roots as well as substantial distribution within the plant when these elements are increased in the nutrient solution. The data also show several cases of interference in accumulation of one element by another. The form of nitrogen also appreciably affected the behavior of several of these metals.

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STUDIES ON THE CHEMISTRY OF THE LIVING BARK OF THE BLACK LOCUST IN RELATION TO ITS FROST HARDINESS. V. SEASONAL TRANSFORMATIONS AND VARIATIONS IN THE CARBOHYDRATES: STARCH-SUCROSE INTERCONVERSIONS[1]

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The seasonal variations in the carbohydrates of the perennial tissues of trees have been often investigated in the attempt to find some clue to their frost hardiness. The products of photosynthesis are stored as soluble sugars and starch in various parts of trees, reaching a maximum value just before leaf drop. When cold weather comes, the starch disappears from the more exposed tissues and often from the entire tree and is not to be found again until warm weather arrives in spring. The relationships between temperature and the form of carbohydrate present vary from species to species and also with different climates (**6, 16, 19, 22, 30, 33**). LEVITT (**21**) has reviewed a large number of papers dealing with this subject and has attempted to correlate the carbohydrate variations with frost hardiness.

The present investigation grew out of a similar attempt to assess the relationship of variations in the carbohydrate constituents of the living bark of the black locust tree (*Robina pseudo-acacia* L.) to the variations in frost hardiness.

The variations and transformation in the carbohydrate constituents of the bark, including reducing sugars, sucrose and starch, were followed seasonally in normal trees for a year and a half. In addition, to determine whether, and to what extent, transformations and variations in the carbohydrates of the bark were independent of the normal seasonal conditions, samples were taken for analysis at various times throughout the year from sections of the bark of trees in which translocation had been intercepted by previous ringing above and below the sampling area. Such unseasonal and independent transformations were also produced and examined in logs cut from trees with the bark intact and stored at different temperatures in the laboratory.

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Materials and methods

The black locust trees used in this study were found on a small hill near the St. Paul campus of the University of Minnesota. The trees were from five to eight inches in diameter, about 15 years old, and had straight trunks, sometimes 10 to 12 feet of the trunk being free of branches. The tissue which was analyzed in this work was the living or inner bark. The brown outer dead corky bark was removed from the trunk with a knife to reveal the light-colored living bark, which would average about 3 mm. in thickness. The living bark was divided into samples of from 0.3 to 0.7 gm. fresh weight, depending on the thickness of the bark and the area of the section, and was removed from the tree. During the spring, summer, and early fall the living bark was easily removed from the tree because of the weakness of the cambium layer, but during the dormant season the sections stuck tightly to the wood, making it difficult to remove them completely. The area from which the bark was removed was about 70×120 mm., small enough so that one tree could be sampled a number of times up and down the trunk without seriously affecting the normal functioning of the remaining phloem.

The samples were weighed and stored in small vials. Two sections were dried at 95° C for six hours in a vacuum oven to determine the per cent. of dry weight of the tissue. The figures obtained were then used to calculate the dry weight of the adjoining sections taken from the same tree. The sections to be used for carbohydrate determinations were stored in 80% alcohol at 30° C. Some of the samples were preserved by first pouring the hot alcohol over the sample and holding the temperature at 70° C for half an hour to destroy enzyme activity, but it was found that no changes in carbohydrates occurred during a five-month storage period even when this pre-heating was not used.

Analytical procedures

Reducing sugars and sucrose were determined by the method of Hassid (**13, 14**). In preliminary studies on the seasonal variations of these constituents of the bark of black locust (**28**), the method of Blish and Sandstedt (**3**) was employed. Values obtained by this method, while showing the same general trend as those found in the present, more extensive study, were consistently higher, which was due, apparently, to a partial hydrolysis of other carbohydrate constituents under the acidic conditions of extraction required by this method (**18**).

Each sample was cut into small strips, placed in a large test tube, and extracted three times with hot 80% alcohol. The extract was then boiled, with small additions of water, until the alcohol was removed and was clarified by adding in succession, 1 cc. of saturated lead acetate, 2 cc. of saturated disodium phosphate, and 0.6 gm. of bone charcoal. Animal charcoal must be used, because plant carbons have been found to adsorb sucrose (**2**). The treated extract was then filtered through a layer of tale on a Buchner funnel and diluted to volume. The reducing value of the clarified extract

was determined by the ferricyanide-ceric sulphate titration method, using Setopaline C as the indicator. Sucrose was determined by hydrolyzing an aliquot of the clarified extract with invertase and determining the increase in reducing power by the same method.

The residue from the alcohol extraction was washed with ether, air-dried, and stored at 3° C until the starch determination could be carried out. Starch was determined colorimetrically using the method suggested by NIELSEN (**24, 25**) and by PUCHER *et al.* (**27**). Each sample was ground in a mortar with sand and 5 ml. of 4.7 *N* perchloric acid. (It is believed that Nielsen made an error in calculating or reporting his perchloric acid concentration for optimum extraction as 4.8 molal instead of 4.8 molar.) A thorough wet grinding was needed to crush all of the starch-containing cells in the tough fibrous bark. The usual preliminary solubilizing treatment with boiling water was not used because other materials were released into solution upon subsequent grinding which caused turbidity and interfered in the colorimetric determination. After grinding and extracting for 15 minutes, the sample was washed into a small centrifuge tube, centrifuged briefly, and the extract was decanted and collected. The residue in the tube was washed with water, centrifuged and the washings combined with the first extract. The sample was then returned to the mortar and extracted again with 5 ml. of 4.7 *N* perchloric acid along with more grinding. This extraction was carried through exactly as the first and the extract was combined with it. A third extraction with perchloric gave complete removal of the starch. The combined extracts were made up to a volume of 50 ml. and could be stored for as long as six days at 3° C without appreciable change.

Starch was estimated colorimetrically as the starch-iodine complex. One milliliter of the extract was tested on a spot plate with an I_2-KI solution to determine roughly the aliquot and dilution required for the colorimetric method. Usually aliquots of 1 or 2 ml. were transferred to a volumetric flask and diluted to 10 to 15 ml. volume. For extracts containing little starch, aliquots of 3 ml. or 5 ml. were used. A drop of phenolphthalein was added followed by 2 *N* NaOH added dropwise until the solution became pink. After back titration to colorless with 2 *N* acetic acid, 2.5 ml. of the acetic acid were added in excess, then 0.5 ml. of a fresh KI solution followed by 2.0 ml. of 0.025 *N* KIO_3. A few minutes were allowed for the color development, and the solution diluted to volume, 25 ml. or 50 ml., depending on the amount of starch present. The solution was then centrifuged briefly and the per cent. of transmission at 590 mμ was determined against a reagent blank. The amount of starch present was estimated by referring to a standard curve prepared with potato starch. The point of maximum absorption for both the potato starch standard and the Robinia bark starch was 590 mμ.

The colorimetric determination of the starch-iodine complex proved to be satisfactory and convenient for the determination of starch in the tissues tested, but certain fundamental drawbacks of the colorimetric method must

be recognized (**29**). The starch used for a standard may have a different absorption maximum or may contain different ratios of amylose and amylopectin than the unknown starch. In this case the absolute amount of starch in the unknown would not be determined, but the relative values, which are important in the study being carried out, would be correct. Another error could result if the amylose-amylopectin ratio were to change seasonally in the tissues being studied.

The takadiastase method suggested by DENNY (**10**) was tried on this tissue, but proved unsatisfactory. One sample of bark which showed no detectable blue stain with iodine contained 2.7% starch according to the takadiastase method, while the colorimetric method gave 0.2% starch.

Qualitative analyses of the soluble sugars by paper chromatography according to the method of PARTRIDGE and WESTALL (**26**) were made on some of the alcoholic extracts. The solvent employed was butanol-ethanol-water and the sugar spots were developed with ammoniacal silver nitrate.

Results and discussion

NORMAL TREES

Bark tissues from 20 trees were used in this study and more than 40 groups of samples were taken between May 1949, and August 1950. Twenty-one were taken between May 20, 1949, and November 29, 1949, while 13 more were taken in the ten-week period between March 30, 1950, and June 12, 1950. Two samples from each group were analyzed for carbohydrates and the analysis was repeated if the two samples did not check to within 10%. The normal variations in carbohydrates between two adjacent sections of the living bark rendered it impossible to obtain greater accuracy.

Reducing sugars, sucrose, and starch, calculated as percentage of the dry weight of the bark tissues, are given in table I together with the dates of sampling and a designation of the trees from which the samples were taken. Trees A to I, inclusive, were cut down at the time of sampling. Trees 1 to 6, inclusive, were ringed initially at the time the samples were collected. Trees 7 to 11, inclusive, were sampled repeatedly for carbohydrate analysis in the manner described above.

The average seasonal variations of the reducing sugars, sucrose, starch, and the summation of these, denoted as total reserve carbohydrates, in the bark of normal trees throughout the year are presented in figure 1. The analytical figures for most of the trees are reasonably close to the smooth curves as shown in the figure, but in an occasional tree the deviation from the average pattern is somewhat more than the experimental error of the determination.

Paper chromatographic analyses performed on extracts of samples collected on February 6, 1950, and July 7, 1950, showed sucrose to be present as the principal sugar, with glucose and fructose present in small quantity together with a small amount of maltose. The extract of a sample taken May 27, 1950, however, showed that no fructose was present even though

TABLE I

DATES OF COLLECTION AND ANALYSES FOR REDUCING SUGARS, SUCROSE, AND STARCH CONTENTS OF THE LIVING BARK OF NORMAL BLACK LOCUST TREES IN 1949 AND 1950. CARBOHYDRATE ANALYSES ARE GIVEN AS PER CENT. OF DRY WEIGHT OF THE BARK TISSUES.

1949 Dates	Tree	Reducing sugars	Sucrose	Starch	1950 Dates	Tree	Reducing sugars	Sucrose	Starch
May 20	A	0.9	1.8	2.2	February 6	9	3.2	9.6	0.1
June 13	B	1.1	3.4	0.5	March 3	8	2.3	7.8	0.1
June 21	C	1.1	3.5	0.5	March 30	9	2.6	8.9	0.3
July 5	1	1.1	5.2	0.4	April 13	9	1.7	9.6	0.5
July 5	2	1.1	5.0	0.4	April 20	9	1.0	7.9	3.2
July 25	3	1.0	6.5	1.1	May 1	9	1.1	7.2	2.7
July 25	D	1.0	5.8	1.2	May 10	7	1.3	6.1	3.4
August 15	4	1.1	6.2	1.7	May 11	1	1.3	7.0	2.4
August 15	5	0.9	4.8	1.5	May 15	9	1.0	5.3	5.8
August 15	E	0.9	7.0	4.4	May 19	7	0.8	4.9	5.6
August 24	6	0.9	8.3	3.3	May 24	9	0.8	3.7	6.7
August 29	F	0.8	10.6	5.5	May 27	7	1.3	1.8	4.7
September 9	G	0.9	9.8	4.8	May 31	9	0.9	1.8	5.8
September 16	7	0.9	10.7	4.3	June 6	7	0.9	2.1	2.6
September 21	H	0.8	8.3	6.8	June 12	7	1.0	2.1	1.0
September 29	7	0.8	11.4	5.8	June 22	9	1.0	3.0	0.9
September 29	8	0.8	10.7	6.0	July 7	7	1.1	5.3	0.9
October 6	9	0.9	9.9	5.8	August 23	10	0.9	11.2	4.0
October 21	8	0.8	10.0	7.9	August 29	10	0.9	10.9	4.3
October 21	11	0.9	7.1	4.0					
November 4	11	0.8	10.2	1.7					
November 28	8	1.4	10.8	0.7					
November 28	9	1.9	11.1	0.8					

the amount of reducing sugar, 1.3% by quantitative analysis, was somewhat larger in this sample than the average for that time of the year.

These chromatographic analyses and the results of quantitative analyses given in table I show sucrose and starch to be the major carbohydrates present in the living bark of Robinia. Tracing first the course of seasonal variations in sucrose throughout the yearly cycle of 1949–1950 beginning in the spring of 1949, it is seen that sucrose was at the minimum level of 2% in early May of that year, having been depleted by the demands of rapid terminal and radial growth which had occurred just prior to that time without compensatory assimilation. Following emergence of leaves and initiation of photosynthesis, the sucrose began to increase. It reached a maximum value of about 11% as early as the last week in August for some trees and remained at this level throughout the fall. By the end of winter the sucrose level had fallen slightly, probably because of respiration. Then in April it began to fall much more rapidly as warmer weather came and this drop was associated with a concomitant and proportionate increase in the starch. The decrease in sucrose and the concomitant increase in starch was slowed somewhat by periods of cool weather, but when the higher temperatures persisted in late May and growth was initiated both sucrose and starch again decreased rapidly to minimum values.

Reducing sugars were at a constant low level, of 0.8% to 1.1% of the dry weight, during most of the year. In late winter they reached a maximum of about 3.0% of the dry weight. Some of the increase may have come from the starch which disappeared with the coming of cold weather, but some may have come from sucrose which also decreased during the winter.

Starch was also depleted initially in late May of 1949 by the flush of new growth, then remained at a summer minimum of less than 1% of the tissue dry weight during June and July. It did not accumulate to any marked extent in the bark until after the sucrose had reached a level of 5 to 6%, but increased progressively during September and reached a maximum in one tree of 8% on October 21, 1949, after leaf drop and just before the first killing frost. Probably some of the carbohydrates, moving out of the leaves

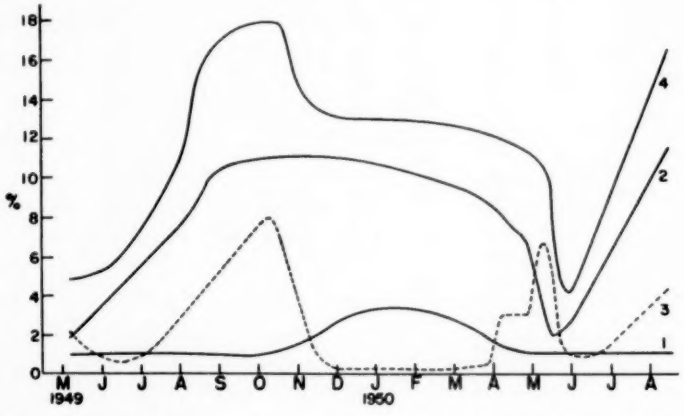

FIG. 1. Average seasonal variation as per cent. of dry weight of tissue, in reducing sugars (curve 1), sucrose (curve 2), starch (curve 3), and total reserve carbohydrates (curve 4) in the living bark tissue of 12 Robinia trees during the period from May 1949 to August 1950.

into the bark of the trunk and branches before leaf abscission, contributed to the total accumulation of starch in the bark which occurred at this time (**23**). Unfortunately the next samples from most of the trees were not taken until November 28. Under the influence of cool temperatures in the intervening period from October 21 to November 28, the starch in the bark decreased from the early autumn maximum to a value of less than 1% and finally reached values in February as low as 0.1%. The analyses performed on November 28 indicated that no increase in sucrose or reducing sugars was associated with the sharp drop in starch content, with the result that a loss of total carbohydrate seemed to have taken place. A probable clue to the fate of the disappearing starch and total carbohydrate at this time in normal trees was obtained in the instance of only one tree either because in this instance sucrose was not at a high level of concentration initially or because only a brief period was permitted to elapse without sampling be-

tween the time of considerable accumulation and the time of disappearance of starch after incidence of cold weather. This tree when sampled on October 21, 1949, contained 4% starch and 7% sucrose in the living bark, but when sampled again on November 4 it had 1.7% starch and 10% sucrose. A considerable percentage of the starch in this tree had apparently been converted to sucrose in this short interval with the advent of cool weather. It would appear, on the basis of this result and of the results on isolated bark segments to be presented later, that in the other trees the starch may also have been converted to sucrose in the fall but that a physiological limit exists in the amounts of sucrose which can be concentrated in the cells or tissues of the bark. Sucrose in excess of this limit, which appears to be roughly 12%, was either not retained in the bark tissue and diffused rapidly out of the bark into the adjoining wood as it was formed or was rapidly transformed into another carbohydrate not included in the analysis. It is probable that sampling and analysis of the bark performed at regular and frequent intervals subsequent to the onset of cool weather might have revealed such transformation of starch to sucrose in the other trees as well.

In early April with incidence of short periods of higher temperatures, a very small amount of starch reappeared in the tissues. Between April 13 and April 20, 1950, there was a three-day sustained period in which the temperature never fell below freezing and during this time the starch content increased from 0.50% to 3.2%. The next three weeks were cold with the temperature dropping below freezing at least every night and no further increase in starch was manifested. Between May 11 and May 15 there was another period of three to four days during which the temperature never dropped below 5° C and again the starch content of the bark rose from 2.4% to 5.8%. With persisting higher temperatures starch finally reached a maximum of 6.7% on May 24 in the bark of one tree along with 3.7% sucrose and 0.8% reducing sugar for a total of 11.2% carbohydrate. The same tree had had 0.3% starch, 8.9% sucrose and 2.6% reducing sugars in the bark on March 30 for a total of 11.8%. During this period of two months with sufficiently prolonged spells of warm weather no considerable change in the total carbohydrates had occurred but the soluble sugars were replaced almost quantitatively by starch. The maximum amount of starch in the spring occurred on May 24, about the time the new leaves were beginning to appear. Three weeks later, when the leaves were fully grown and a considerable portion of the new wood had been formed, the starch, along with sucrose, had dropped to summer minima. The yearly cycle was now complete.

The spring of 1950 was the coolest in 50 years in Minnesota and this was reflected in the delayed seasonal growth in the locust and therefore in the variations in its bark carbohydrates. In the spring of 1949, which was a normal one, starch and sucrose had each been depleted by growth to a level of approximately 2% on May 20, while in the spring of 1950 growth was not sufficient to deplete the starch and sucrose to this level until June 6. However, by the first week in July 1950 the stored carbohydrates were the same

as the 1949 values. This delay in seasonal activity observed in the cool spring of 1950 is in accord with the findings of DAUBENMIRE (9) who reported that Robinia showed no increase in the diameter of the trunk in those periods in the spring when the minimum temperature remained below – 1° C. A delay in budding and flowering of a number of trees and shrubs in the same area was also observed during the spring of 1950 (17).

The complete disappearance of fructose (as indicated in the chromatographic analyses of extracts of May 27, 1950) in the spring at the end of the period during which sucrose is metabolized most rapidly is significant and may indicate some peculiarity in the metabolism of the tree at this time.

LOGS CUT IN SPRING

The above analyses of the seasonal variations in the starch and sucrose contents of normal locust bark tissue reveal the following approximate interrelationships: (a) Starch begins to accumulate in summer only after the sucrose content has reached a value of approximately 3 to 4% of the dry weight of the tissue. (b) When the sucrose content reaches a value of 10 to 12% of the tissue dry weight, further accumulation of assimilable carbohydrate is entirely in the form of starch. (c) When autumnal temperatures approach the freezing range, starch quickly and almost completely disappears from the tissues. If the sucrose content is much below the apparent limiting value of 10 to 12%, a part of the starch loss will be reflected in an increase in sucrose to a value approximating this limit. Carbohydrate derived from starch in excess of the amount needed to bring sucrose to its limiting value is lost from the analyses, due possibly to its diffusion (as sucrose or other soluble carbohydrate) into the wood of the trunk or its conversion to hemicellulose or other form not determined in the analyses. (d) Sucrose content remains high through the winter. When spring temperatures remain well above the freezing point, the sucrose present (perhaps that in excess of the 3 to 4% required under (a)) rapidly disappears and a nearly equivalent amount of starch reappears in the tissue. Both then quickly drop to summer minima as the growth of new tissue occurs.

These observations suggested that a reversible temperature dependent upon sucrose-starch interconversion mechanism exists in the living bark tissue of the locust tree and prompted an effort to convert the starch, at the point of its highest accumulation in spring, back to sucrose by artificial application of low temperature.

Three lengths of log with bark intact were cut from a tree on May 22, 1950, when the spring accumulation of starch was near the maximum. Samples taken from the living bark of one log were immediately frozen for preservation and later analysis. A second log was transferred to a refrigerator at + 3° C where respiration would be depressed, and the third log was placed in the laboratory subject to the normal temperature fluctuations usually between 20 and 24° C. A month later the bark of the second and third logs were sampled and analyzed along with the frozen samples taken

from the first log. The results of these analyses and those obtained on a normal tree on June 22 are shown in table II. The water contents of the bark tissues at the times of sampling are also indicated.

The cut log kept at room temperature dried out somewhat and no cambial division or deposition of wood was observed to have taken place during the period of storage. Respiration, however, must still have been maintained at a high level because considerable depletion of carbohydrate occurred. Although sucrose and the reducing sugars were originally the readily available carbohydrates, their concentration did not change during this period; the starch stored in the bark, therefore, seemed to have furnished the metabolized carbohydrate. It is possible that starch is used more readily in respiration and anabolic processes than the free reducing sugars or sucrose. The replacement of sucrose by starch prior to active growth in spring would suggest that starch rather than sucrose was to be utilized as the immediate substrate. The identification of an active phosphorylase in locust bark which converts starch to glucose-1-phosphate is consistent with this view.

TABLE II

VARIATIONS IN THE CARBOHYDRATES AND WATER CONTENT OF THE LIVING BARK OF ROBINIA DUE TO CHANGES IN TEMPERATURE. EXPRESSED AS PER CENT. OF DRY WEIGHT OF BARK TISSUE.

	Reducing sugar	Sucrose	Starch	Total	Water content
	%	%	%	%	%
May 22, original	0.9	2.7	7.0	10.6	110
June 22, 3° C storage	1.3	8.5	0.2	10.0	98
June 22, 20 to 25° C storage	1.0	2.9	0.2	4.1	64
June 22, normal tree	1.0	3.0	0.9	4.9	164

The variations in carbohydrate in the bark of the normal tree, exposed to approximately the same temperature conditions outdoors as prevailed in the laboratory, appeared to have followed a course from May to June similar to those of the cut log stored in the laboratory. This similarity was more apparent than real. The bark of the normal tree was highly hydrated and also considerable radial and terminal growth with active deposition of wood had taken place in this tree in the interval from May to June. Thus the depletion of carbohydrate in this normal tree must have exceeded that of the stored log. However, assimilation in the normal tree, following emergence of leaves at this time, must have compensated for the extra depletion so that fortuitously similar levels for total carbohydrate were finally established on June 22 in the bark of the normal tree and in that of the cut log.

In contrast to these results, the cut log transferred on May 22 to the refrigerator at +3° C, where both respiration and growth were depressed, showed little depletion of carbohydrate but at the same time a very nearly quantitative replacement of starch by sucrose. The starch which was here apparently converted to sucrose had accumulated originally in the intact

tree in May with a simultaneous decrease in sucrose. The reversibility of these transformations under the influence of temperature is indicated. The equivalence also of starch and sucrose in these transformations both in normal trees and in the cut logs would imply that direct interconversions of starch and sucrose may take place in the bark of the locust tree.

Trees ringed in summer and fall

To determine the variations which occur in the carbohydrates as a result of impeded translocation, two 1.5-inch rings two to three feet apart were cut through to the wood of a number of trees at various times during the summer of 1949. At intervals during several weeks new rings were cut two to three feet above the previous rings until some trees had as many as six rings, all on the straight portion of the trunk below the branches. Curtis (**7, 8**) has shown that cutting the phloem arrests the transfer of carbohydrates and other organic nutrients, so that the sections of living bark isolated between rings were deprived of fresh supplies of these nutrients. Swarbrick (**31**) noted that in apple trees, ringing injured only the very outer portions of the xylem so that water conduction was not impaired. In the locust on the other hand all but the newest annual xylem rings are plugged by tyloses (**11**) leaving much less functional xylem. Trees ringed after the middle of July, however, did not wilt and the moisture content of the bark above the rings was very similar to that of normal trees. Evidently sufficient new xylem had been laid down previous to this time to permit normal water movement past the rings even though the xylem at the point of ringing was exposed to the air. Partial yellowing and dropping of the leaves did occur in some trees, particularly in those in which ringing was initiated early in the summer. The remaining leaves were healthy and green and did not wilt. Also photosynthesis in these leaves could not have been impaired because carbohydrates continued to accumulate in the bark of the trunk above the rings.

Samples of the bark were taken for carbohydrate analysis from the bark area near each ring, or from the ring itself, at the time of its excision from the trunk. In the ringing and cut log experiments, it is to be emphasized that differences in the analytical values existing at any time between samples taken from different levels on widely separated parts of the trunk of an intact tree are never greater than the differences encountered between adjacent samples. Any differences which arise following the isolation of sections by ringing or cutting and which exceed the approximately 10% variation between adjacent samples must therefore be considered as significant and resulting from the isolation procedure. The analytical values found in the bark of samples near or from the first two rings represent normal tree values at the time of cutting of the rings while the values found in the bark of samples taken near or from rings cut subsequently above the earlier rings on the trunk of the same tree indicated the extent of accumulation of carbohydrates in the bark that remained in phloem communication with the leaves. These analytical results are shown in table III, under the columns headed A. The trees were cut down and samples were taken again

TABLE III

CARBOHYDRATE ANALYSES ON SECTIONS OF THE LIVING BARK OF RINGED ROBINIA TREES. SECTION 1 IN EACH CASE WAS ISOLATED ON THE TRUNK BY PLACING RINGS ABOVE AND BELOW IT ON THE DATE INDICATED. SUCCESSIVE SECTIONS WERE ISOLATED BY PLACING A SINGLE RING HIGHER ON THE TRUNK ON THE DATE GIVEN. ANALYSES OF THE SEGMENTS AT THE TIME OF ISOLATION (A) AND IN MID-NOVEMBER (B) ARE EXPRESSED AS PER CENT. OF THE DRY WEIGHT OF THE TISSUE.

Tree	Section analyzed	Date of ringing	Reducing sugar		Sucrose		Starch		Total	
			A	B	A	B	A	B	A	B
			%	%	%	%	%	%	%	%
2-R										
	Crown			1.0		7.1		0.3		8.4
	2	August 24	0.8	1.0	8.4	6.8	3.5	0.2	12.7	8.0
	1	July 5	1.1	....	5.2	....	0.3	....	6.6	....
	Stump		1.1	1.1	5.2	9.0	0.3	0.6	6.6	10.7
3-R										
	Crown			0.8		7.4		3.0		11.2
	5	September 21	....	0.8	....	7.8	....	3.1	....	11.7
	4	September 8	....	1.0	....	10.2	....	2.7	....	13.9
	3	September 2	0.7	0.9	7.2	9.5	11.5	4.7	19.4	15.1
	2	August 15	1.0	1.0	7.1	6.0	14.4	0.5	22.5	7.5
	1	July 25	1.0	1.1	7.5	4.3	1.2	0.2	9.7	5.0
	Stump		1.0	1.0	7.5	9.5	1.2	0.8	9.7	11.3
4-R										
	Crown			0.8		9.9		5.7		16.4
	4	September 21	0.7	1.0	6.3	9.2	15.6	3.4	22.6	13.6
	3	September 2	0.9	1.0	7.7	7.1	10.0	2.0	18.6	10.1
	2	August 24	0.9	1.1	9.5	6.5	8.3	0.9	18.7	8.5
	1	August 15	1.1	1.1	6.2	3.5	1.8	0.8	9.1	4.9
	Stump		1.1	1.0	6.2	6.5	1.8	0.3	9.1	7.8
5-R										
	Crown	October 6*	0.7	0.9	5.4	11.4	16.0	5.3	22.1	17.6
	3	September 9	0.7	0.9	7.2	8.9	11.6	1.9	19.5	11.7
	2	August 24	....	1.0	....	6.7	....	0.6	....	8.3
	1	August 15	0.9	0.9	4.8	3.7	1.5	0.2	7.2	4.8
	Stump		0.9	1.1	4.8	9.0	1.5	0.6	7.2	10.7
6-R										
	Crown			0.7		12.2		7.4		20.3
	4	September 21	0.7	0.8	5.8	11.7	15.4	4.9	21.9	17.4
	3	September 9	0.8	0.9	8.4	10.4	10.0	3.4	19.2	14.7
	2	September 2	0.8	1.0	10.0	9.1	5.7	1.4	16.5	11.5
	1	August 24	0.9	1.0	8.4	8.2	3.3	0.8	12.6	10.0
	Stump		0.9	1.1	8.4	9.2	3.3	0.7	12.6	11.0

*A sample was taken from above the highest ring.

in the middle of November. Samples were taken from above the highest ring (crown), from below each ring, and from below the first ring (stump). The analyses of these samples are found in table III, columns B, and are listed beside the value of the corresponding sample taken at the time of

ringing so that the changes in carbohydrate between the time of ringing and November may be seen for each section of the trunk.

SWARBRICK (**32**) reported that starch accumulated rapidly above rings in apple trees if these rings were cut after the tree was in full leaf. Starch also accumulated very rapidly above the rings in Robinia, and by late September the crown of some ringed trees had 16% starch in the bark, as compared to 6 to 7% in normal trees at this time. The longer the duration of the period of communication of a section with the leaves before being cut off from above by a new ring the greater was the accumulation of starch in the section. The accumulation, therefore, was most marked in the crowns and upper sections of the trunk.

The sucrose in the bark of areas above newly cut rings in some cases increased during the first week or two after ringing, but after this period a decline was observed. The amount of reducing sugar above the rings always decreased after ringing but the difference was slight and similar to that which occurred in normal trees during this period. The total carbohydrate, largely in the form of starch, was always much greater above the rings than in normal trees.

One of the primary effects of the ringing in summer when the trees were in full leaf was the abnormally high accumulation of carbohydrate in the bark above the rings. In the sections which remained longest in phloem communication with the crown, starch became the major storage carbohydrate instead of sucrose. Another effect in the isolated sections was to prevent any loss or gain of carbohydrate by translocation through phloem to or from the leaves or root subsequent to the time of isolation. Any fluctuations which occurred therefore in the carbohydrates of these sections were due either to inter-transformations within the existing carbohydrates or to losses entailed by respiration, diffusion into the wood, or conversion into carbohydrates not included in the analysis. It is evident generally in the results shown in table III that ringed sections did contain less total carbohydrates in November than when the rings were cut, though differentiation between transpirational and other possible pathways of these losses is not clearly indicated in the analyses.

The most conspicuous changes observed in a number of the later ringed sections were those involving the replacement of the accumulated starch by sucrose. For example, the bark of the section ringed October 6 of the no. 5–R tree contained 16% starch on October 6 and 5.3% starch on November 17 while the sucrose content increased from 5.4 to 11.4%. A striking illustration was therefore provided, in these ringed sections, of the simultaneous decrease in starch and increase in sucrose as induced by low temperatures. However, the conditions required for such transformations to be readily observed, even in ringed trees, were established in only a limited number of sections. The conditions required were, first, the total carbohydrate level in the bark at the time of ringing had to be in considerable excess of the amount which would subsequently be lost by respiration or diffusion prior to the onset of cold weather and, secondly, this carbohydrate had to be pres-

ent, initially, largely in the form of starch rather than sucrose. In normal trees, where sucrose is the major storage carbohydrate, a maximum level of 10 to 12% sucrose is attained in September. This appears to represent a physiological limit for sucrose concentration in the bark of normal trees. Any excess is lost by diffusion into adjacent wood or by conversion to other and undetermined carbohydrates. Thus, because this excess sucrose is not retained in the tissue, starch to sucrose conversion in normal trees was observed in only one instance. A similar limit seems to have been established in the sections of ringed trees. In no case was a sucrose level greater than 12.5% encountered. Because of the predominant storage of starch rather than sucrose in these ringed trees, however, the levels of sucrose were initially low enough to afford ideal circumstances for the conversion of starch to sucrose to be detected, in spite of any losses of total carbohydrates which occurred at the same time. Thus the increase in sucrose at the expense of starch occurred in those bark sections which, having been early intercepted from below by rings but not from above until much later, had accumulated total carbohydrates in considerable excess over that present in normal trees and which had also stored this carbohydrate in the form of starch rather than sucrose.

The basal sections of the ringed trees (below the first rings), which were cut off from the leaves but not from the roots, in many cases contained more carbohydrates in November than when the rings were cut. They also contained more carbohydrates at this time than the first ringed section of the bark above them which had been cut off from the crown and leaves at the same time. The final concentration of carbohydrate was remarkably uniform in almost all of these basal sections, ranging between 9.2% and 11.4% in 11 of 12 ringed trees regardless of whether the rings above them had been cut in July or late August. The carbohydrate in these basal sections may have been derived either from adjacent trees via interconnecting root systems (which is often observed in locust clones) or from carbohydrate stored in the roots.

Trees ringed in winter

The effects of cutting the rings in winter on the subsequent transformations which occur in the carbohydrate constituents of the bark of the locust tree were also examined. Two trees were doubly ringed in the late winter of 1950 and the results of analyses for carbohydrate in samples of the bark taken from above and below the rings at the time of ringing and also later on June 23 are shown in table IV. These trees were ringed long before any radial or terminal growth was initiated. If the previous year's xylem had become plugged with tyloses as is reported to occur in the locust (**11**) ringing of the bark would not only arrest translocation in the phloem but would, by destruction of the cambium at the points of ringing, prevent the formation of new xylem and thereby impede the normal upward transport of water in the trunk from the roots. This was indeed indicated by the results of the moisture determinations made on these ringed trees, as given in table IV. The bark of those parts of the trunk situated above the rings had, in June,

TABLE IV

CARBOHYDRATE AND MOISTURE ANALYSES ON SECTIONS OF THE LIVING BARK OF ROBINIA TREES AT THE TIME OF AND FOLLOWING DOUBLE RINGING OF THE TRUNKS IN WINTER. ANALYSES ARE EXPRESSED AS PER CENT. OF THE DRY WEIGHT OF THE TISSUE.

	Reducing sugar	Sucrose	Starch	Total	Water content
	%	%	%	%	%
8-R tree					
March 3, 1950, ringed	2.3	7.8	0.1	10.2	86
June 23, 1950, above rings	2.0	9.6	0.4	12.0	86
June 23, 1950, between rings	3.3	3.1	0.5	6.9	84
June 23, 1950, below rings	1.2	2.8	4.8	8.8	145
9-R tree					
February 6, 1950, ringed	3.2	9.6	0.1	12.9	119
June 23, 1950, above rings	0.9	6.1	1.3	8.3	63
June 23, 1950, below rings	1.3	1.9	4.4	7.6	151
June 23, 1950, normal tree	1.0	3.0	0.9	4.9	164

a much lower water content than normal trees at this time. The effects, then, which were observed on these trees in late spring and early summer, of being ringed in winter could be traced in part to the restrictions in water supply. Budding and cambial division and the growth of new wood which normally follow these processes was completely arrested in the crown and trunk of the tree above the rings. As a result, the carbohydrates in these sections were not depleted to the extent that they were in normal trees at this time. In addition even at the high temperatures prevailing in June, little or none of the normal replacement of sucrose by starch had occurred although a good supply of sucrose had been retained. It is thereby indicated that initiation of budding, cambial division and the process of apparent conversion of sucrose to starch in the locust are not only dependent on temperature but also on the state of hydration of the tissues. It is implied therefore that the enzymatic systems in the intact cells which are involved in these processes are dependent on the state of hydration of the cell as well as on the temperature and that the activity of water in the cells may be a factor in the regulation of these systems. It is of interest that in the bark of the basal sections below the rings, which had a high but normal water content for that time of the year, a considerable decrease in sucrose and increase in starch had taken place. At the same time normal cambial division had been initiated and some new wood formed. However, the extent of formation of new wood and of depletion of total carbohydrate in these basal sections were not so great as in the bark of normal trees at this time even though their water content was comparable to that of normal trees. For unknown reasons some delay of the normal seasonal processes had occurred even in these basal sections because of the rings above them.

The results obtained in this investigation indicate that the principal variations in the carbohydrates of the living bark of the black locust (Robinia) tree can be accounted for on the basis of variations in starch and sucrose.

The seasonal variations in the total amounts of these carbohydrates stem from the seasonal periodicity in assimilatory and growth activity of the tree in that these processes determine the extent to which these carbohydrates will be accumulated in the bark after translocation from the leaves or be consumed as energy sources in respiration and as precursors in the synthesis of other carbohydrates such as cellulose. The preferential utilization of starch rather than sucrose as the immediate metabolic substrate in the bark tissues is indicated.

The variations in the relative proportions of the starch and sucrose in the bark tissue are apparently due to a process of interconversion and must be mediated by reversible enzymatic reactions which are controlled in some way by temperature and to some extent also by the degree of hydration of the tissue. The synthesis of starch from sucrose is favored by high temperature and the reverse reaction is favored by low temperature. It is not known whether more than one enzyme is involved in these reactions because the process of interconversion has not been achieved *in vitro*. It is also not known whether simple hexoses or phosphorylated hexoses are intermediates in the conversion. However, the variations in starch and sucrose which are observed in the bark in some instances where growth is at a minimum (early spring and late fall) or where translocation and accumulation are eliminated (cut logs and ringed trees) are such as to indicate that the degree of these conversions is considerable and in some cases almost quantitative. The hypothesis may be advanced that a process of direct conversion of sucrose to starch, and perhaps starch to sucrose as well, operates in the bark of the locust tree, though the latter process appears less plausible on energetic grounds. An enzyme capable of converting sucrose to starch has been extracted from bacteria (**15**). The studies of Bois and Nadeau (**4, 5**) would indicate that such a direct conversion process operates in the sap of the sugar maple although others have not been able to confirm their work (**12**). Kursanov and Pavlinova (**20**) have reached the conclusion that the nearest precursors of sucrose in plants are not phosphorylated sugars nor glucose and fructose but compounds with 1,4 α-glucoside linkages such as maltose, dextrins, and starch. The results reported in the present paper could yield support to their contention.

The mechanism whereby temperature controls the direction of these conversions in the bark of the locust is obscure. Arreguin and Bonner (**1**) have investigated the mechanism of starch-sugar transformations in the potato as influenced by temperature and presented evidence that these processes are controlled through the action of a starch phosphorylase inhibitor which is elaborated in the potato tissue at higher temperatures.

Summary

Sucrose and starch are the principal storage carbohydrates in the living bark of the black locust. Both carbohydrates are depleted in late spring by the flush of new growth. Sucrose begins to accumulate again shortly afterwards and reaches a maximum in late summer. Starch does not appear in

the bark as a storage product until late in the summer and reaches a maximum in early fall but the maximum content is not as high, in normal trees, as is that of sucrose. Starch disappears with the coming of cold weather in late fall but sucrose is retained in high concentration throughout the winter. In the instance of one intact tree where sucrose accumulation was abnormally low, it was found that the disappearance of starch is accompanied by a concomitant rise in sucrose concentration.

With the advent of warm weather in early spring and prior to initiation of growth, sucrose appears to be quantitatively converted into starch. This conversion occurs only when the daily temperature has a minimum above 0° C. Artificial application of sustained low temperature (3° C) to bark tissues at this time causes the starch to be converted quantitatively back to sucrose.

Reducing sugars are present in low amounts throughout the year, reaching a maximum in late winter. Fructose is absent in late spring during the period of most active growth.

Trees ringed in summer accumulate much more carbohydrate in the bark above rings than do normal trees at this time and, in contrast to normal trees, this excess carbohydrate is present largely in the form of starch rather than sucrose. With incidence of low temperature in autumn a readily observable conversion of the starch to sucrose occurs in the later ringed sections of these trees.

Sections above rings of trees ringed in winter show no signs of radial or terminal growth in spring. They do not become hydrated as do normal bark tissues at this time and their carbohydrates are not so radically depleted. The carbohydrates are retained in the form of sucrose and little or no conversion of this sucrose to starch is observed even at the higher temperatures prevailing at this time.

These observations on seasonal and unseasonal variations in the carbohydrates of the black locust suggest that a temperature-sensitive and reversible enzymatic process is operating in the bark of the tissue of this tree which determines the direct conversion of sucrose to starch and perhaps of starch to sucrose as well. It is also indicated that this process may be dependent on the state of hydration of the cells of this tissue.

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INVESTIGATIONS OF NITROGEN COMPOUNDS AND NITROGEN METABOLISM IN PLANTS. III. γ-AMINOBUTYRIC ACID IN PLANTS, WITH SPECIAL REFERENCE TO THE POTATO TUBER AND A NEW PROCEDURE FOR ISOLATING AMINO ACIDS OTHER THAN α-AMINO ACIDS

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The technique of partition chromatography on paper has given a new tool for the identification of constituents of the soluble nitrogen fraction of plants (**8, 18**). It is not surprising therefore that hitherto unrecognized amino compounds are now being revealed by its aid (**10, 12, 15, 26, 27**). Among the amino acids now known, or believed to occur free in plants, are some which have been recognized by partition chromatography on paper (*e.g.*, β-alanine).

Although γ-aminobutyric acid has long been considered to be a metabolite of bacteria (**1, 2, 3**), it was the advent of paper chromatography that revived interest in this substance and stimulated work which has led to its recognition as a constituent of higher plants and animals. Following the first references to this substance as a constituent of the potato tuber (**8**), it has now been recognized in, and in fact isolated from, the tissue of the beet root (**26**), from yeast (**15**), from brain (**4, 25**), and from rye grass (**21**).

The first complete examination of a plant tissue by two-dimensional paper chromatography for the amino acids was made by DENT *et al.* (**8**) on alcohol extracts of the potato tuber. In this first examination, an unexpected range of the known amino acids was revealed as existing free in the tissue, and there were also several substances, then unidentified, which reacted with ninhydrin. One of the most prominent of these was an acid, no. 23, DENT *et al.* (**8**), which appeared on the two-directional phenol : collidine-lutidine chromatograms in a position immediately below that of valine. Almost at the outset, it was suspected that the substance which occupied the position below valine was γ-aminobutyric acid (AB), because known AB occupied the identical position. More definite proof of the identity of this acid in the potato was announced by STEWARD *et al.* (**19**).

Analysis by the quantitative paper chromatographic method of potato tuber tissue shows that the acid now known to be γ-aminobutyric acid is an important constituent of the alcohol-soluble nitrogen of the tissue. Representative analyses have already been published (**19, 22**) which show that

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the acid in question forms a considerable part (3%) of the total alcohol-soluble nitrogen and, excluding the amides glutamine and asparagine, is one of the amino acids present in largest amount (11% of the soluble nitrogen). Of the free amino acids, only arginine contributes more nitrogen to the soluble fraction than AB.

Various kinds of presumptive evidence lead to the identification of spot no. 23 of DENT *et al.* (8) as AB. Synthetic AB added to potato extracts accentuates the AB spot and shows no sign of being resolved during two-directional chromatography. If the area occupied by AB is removed and the acid extracted, the isolated material chromatographs exactly as does

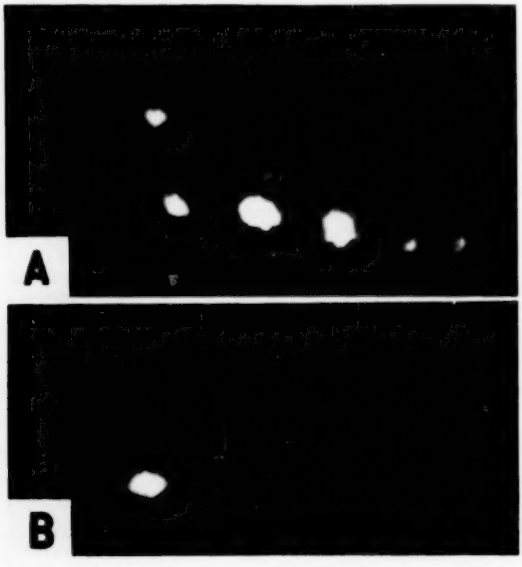
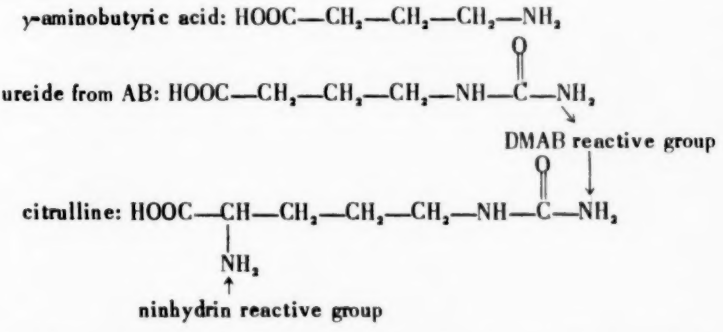


FIG. 1. A. Phenol-collidine chromatogram of soluble nitrogen compounds of potato. B. Phenol-collidine chromatogram of soluble nitrogen compounds of potato in the presence of copper carbonate.

known AB in one-directional or strip chromatograms using a variety of different solvents such as collidine, phenol, butanol, etc. Also, mixtures of known AB and the isolate are not resolvable by chromatography on paper. If $CuCO_3$ is added to the paper along the route taken by the nitrogen compounds moving in phenol, then the amino acids which can form complexes with copper are immobilized and do not move to their customary positions when chromatographed with collidine-lutidine (7) in the direction at right angles to phenol. Figure 1 A shows a normal chromatogram of the soluble nitrogen compounds in potato and figure 1 B a chromatogram in which all the compounds containing α-amino groups have been immobilized and coupled to copper. Only two compounds remain mobile in collidine-lutidine and react with ninhydrin. These compounds are β-alanine and AB.

On being warmed with urea AB forms a ureide which will react with paradimethylaminobenzaldehyde (DMAB) to give a colored compound. Ureides react to give a yellow-orange color while urea gives yellow. The ureide formed by the material as isolated from potato on the papers chromatographs in different solvents in a manner identical with known AB, and mixtures of the two cannot be resolved (**19**). The ureide reacts rather weakly with ninhydrin and presumably has the following constitution in which the reactive groups are designated. The analogy with citrulline (a naturally occurring ureide) will be obvious.



Evidence of the kind indicated above is very strong. Final proof, however, requires the isolation of the chromatographically pure acid from the tissue and some attempt to show, by methods independent of chromatography, that the acid as isolated is identical with the synthetic material. Attempts were made therefore to isolate pure AB from potato tubers and to obtain crystalline preparations and x-ray diffraction patterns to compare with those of the known material. In the interim, evidence for the successful accomplishment of the isolation from yeast had been presented by others (**15**).

The first attempts to prepare AB from potato tuber, by methods very similar to those which were successful on yeast (**15**), resulted in a product which, though clearly rich in this substance, yielded a glassy material from which crystals were very difficult to obtain. One of the pitfalls in paper chromatography is that some quite unrelated substances do overlap. In fact, it is now known that, under the chromatographic conditions used here, AB and δ-aminovaleric and β-aminoisobutyric acids are almost exactly superimposable. It quickly became clear that the difficulty of crystallizing the isolated AB far exceeded that of obtaining crystals of the synthetic substance, even when they were both present in comparable amounts. X-ray diffraction patterns of the crystals, so obtained, were similar to those of pure AB but were not exactly identical with them (fig. 2). For this reason, the full description of the isolation of a pure product from the potato tuber has been deferred until this question could be finally resolved.

Synthetic AB is most conveniently prepared from the corresponding

lactam which yields AB when it is hydrolyzed by the method of DeWitt (**9**). However, methanol and water make a better solvent for the final crystallization than ethanol and water. When pure, AB does not readily form the lactam unless it is heated above the melting point. It is stable and may be dissolved in water, crystallized and chromatographed without apparent loss.

The isolation from commercial yeast extract by Reed (**15**) was carried out in a large part by partition chromatography on starch, subsequent to the isolation by paper chromatography. The successful isolation from an aqueous extract of beet roots (**26**) was carried out mainly by the use of specified ion exchange resins. By similar methods, Hulme and Arthington (**12**) obtained a product from apples which was chromatographically identical with AB. The isolation from brain (**4**) utilized an alcohol extract which was subsequently fractionated on a starch column and, although enough of the free AB for recrystallization was not obtained, a satisfactory product was isolated as a silver salt. The isolation from rye grass by Synge (**21**) adopted a novel procedure. Using Chibnall's procedure of cytolyzing leaves in aqueous ether, a pressed, filtered, juice was obtained. This juice was dialyzed, and the dialyzate subsequently fractionated by electrodialysis

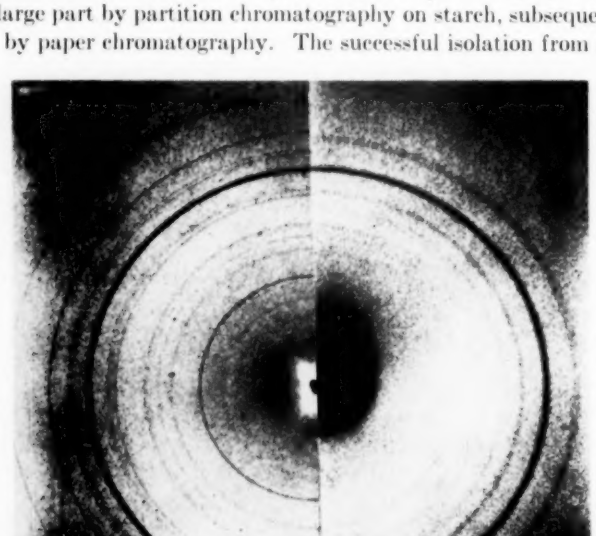


Fig. 2. Left, x-ray diffraction pattern of synthetic γ-aminobutyric acid; right, x-ray diffraction pattern of the isolated material. A small circular disk opening, 0.5 mm. diameter, was fitted on a pin hole system of a universal diffraction camera. A flat photographic film was rotated about the axis of the central x-ray beam. Distance from specimen to emulsion, 5 cm.; Cu target; Ni filter; time of exposure, eight hours.

which enabled the bulk of the α-amino acids to be separated from the others. By utilizing the differential rate of movement of the amino acids in silica gel under the influence of an applied potential, still further purification of the amino acids which have terminal amino groups (AB and β-alanine) was achieved. The final isolation utilized partition chromatography on a cellulose column with butanol-acetic acid and water as the solvents. By these various procedures, AB was isolated in widely different amounts. In fact only the procedure of WESTALL (**26**) which yielded 3 gm. of AB from beet tissue, furnished more than minute amounts (less than 100 mg.) of the final product.

It was thought that the protein synthesis which occurs when potato slices are exposed to aerated solutions of potassium chloride (**17, 20**) caused a relatively greater decrease in the other amino acids than in AB so that the earliest attempts to isolate this material were made on disks which had been so treated for some two to three days. The value of this step is now questioned.

Papers were heavily loaded along one edge with the evaporated alcohol extract of potato tissue and, after chromatography in one direction (phenol), the strip of paper containing the AB was removed and extracted. The procedure was repeated using the second solvent (collidine-lutidine). In this way AB was prepared free from other ninhydrin reactive substances. The amino acids known to be in the tissue and which are the most likely contaminants of AB isolated by this procedure are methionine sulphoxide and β-alanine. The δ-aminovaleric acid or β-aminoisobutyric acid, if present in the tissue, would also accompany the AB. Although solutions were obtained in which there were 25 to 50 mg. of AB, these did not readily yield crystals. On the contrary, the material so isolated showed a strong tendency to set to a glass. This result raised the question whether the chromatographically pure substance free from other known ninhydrin reactive material was in fact a single substance. This question prompted the isolation of AB on a larger scale and for this purpose the paper method was not suitable.

Bulk isolation of the amino acids using the mercuric acetate procedure of NEUBERG and KERB (**14**) was not particularly successful because the AB was even less completely precipitated than the other amino acids. Further trials were made by hydrolyzing the amides in an aqueous extract of potato, removing the dicarboxylic acids as their barium salts and then subjecting the remainder to partition chromatography on starch columns using an automatic fraction collector. Even by these means the yield was very unsatisfactory and the purity of the product was still open to question. The crystals, obtained with difficulty from the glassy material mentioned, yielded the x-ray diffraction patterns shown in figure 2, and these contained lines not attributable to AB.

It is still difficult to understand why these procedures did not isolate a pure product in good yield. It can only be surmised that some other product in the potato accompanied the AB through the various procedures and this

material, present in the final product, accounted for the difficulty in crystallization. In these circumstances an entirely new approach was devised which clearly has merit in that it may be of general application to the isolation of amino acids which are not α-amino acids. The procedure requires no specialized equipment and is simpler than that of SYNGE (**21**).

Materials and methods

ISOLATION OF AB FROM POTATO TUBER IN BULK

Using the method described below, nearly 4 gm. of chromatographically pure AB was isolated from approximately 13 kilograms of potato tubers. The potato tubers were ground in water in a Waring Blendor, the extract was filtered free from starch and heated to coagulate the protein which was then removed. The solution was concentrated by boiling and boiled with acid to hydrolyze the amides. At this point, analysis showed that the extract contained about 8.9 gm. of AB. Free acid (HCl) was removed from the concentrated aqueous extract by repeatedly passing it through an acid-binding amberlite resin (IR-4B of Rohm and Haas) until the solution became alkaline (pH 8.5). The mixed amino acids passed through the column but were accompanied by sugars and most of the soluble products of the tissue. Some of the dicarboxylic acids but none of the monocarboxylic acids remained in the column. A cation binding resin (amberlite IR-120H of Rohm and Haas) was next used to obtain a clean cut separation of the amino acids from all the non-ionizable materials. To do this, the amino acids were absorbed on the resin, which was thoroughly washed with water, and then the amino acids were eluted with approximately 3 N ammonia. Until this point no appreciable enrichment of AB had occurred. Excess ammonia was removed by boiling, and the total extract was concentrated to about 500 ml. At this stage, the extract contained some 5.8 gm. of AB the remainder presumably having adhered to the columns; thenceforeward the yields were very nearly quantitative. To reduce the contamination of the sample with glutamic and aspartic acid, the sample was passed again through the acid-binding resin (IR-4B) which allows the monocarboxylic acids to pass through. At this stage the solution still contained some aspartic and glutamic acid and the remainder of the original amino acids of the tissue. It was freed from ammonia by making it slightly alkaline and boiling.

The special step now adopted utilized the property by which the material in the tissue was shown not to be an α-amino acid, *i.e.*, by its failure to form a complex with copper so that it remains mobile when the α-amino acids are immobile (**7**). To achieve this end in a manner that allowed relatively large amounts of material to be dealt with, a special absorption column was prepared as follows. Basic copper carbonate (azurite), dried and powdered, was mixed with three times its weight of activated alumina (80 mesh) as supplied by Aluminum Company of America. This material was packed dry into three columns, approximately 200 cm. long by 4 cm. in diameter. The

amino acids were put on the column in aqueous solution, and the solution was run successively through three columns until all of the characteristic blue color of the copper complexed amino acids remained on the column. At this stage the effluent was slightly green due to copper salts. After removing the copper with H_2S the only ninhydrin reactive material detected by chromatography in the extract was AB. After most of the AB was crystallized out, traces of other acids were detected in the concentrated residues.

The concentrated solution containing AB was dried, treated with conc. HCl, which dissolves the AB acid but not the sodium chloride which had been added during the preparation. Activated charcoal removed colored material, and the final solution was evaporated to a syrup which crystallized. The crystals of the hydrochloride of AB were separated on sintered glass, dried, and treated in solution with silver oxide to remove chloride and with H_2S to remove silver. Recrystallization was carried out by adding a 1:1 mixture of ethanol and methanol to a concentrated aqueous solution. This procedure yielded a total of 4 gm. of AB, from which 1 gm. of purified, twice recrystallized material for ultimate analysis was prepared.

The method described above by which α-amino acids are combined with copper and separated from other amino acids by chromatography on mixed columns of copper carbonate and alumina clearly has novel features that are of more general application. In considering the further use of this procedure several points may be made. The copper complexing reagent should be an integral part of the column, *i.e.*, one cannot achieve the same end by preparing the complexes and then chromatographing these on alumina alone, for the copper complexes will decompose (**5**). Alternatives to alumina may be found; Fuller's earth can be used; silica gel was not effective. Under the circumstances described α-imino acids, such as proline, adhere to the column but not so tenaciously as the α-amino acids. β-alanine does not form a complex with copper as readily as the α-amino acids, but it does appear to adhere to the column more readily than does AB. This would seem to offer a means of separation of α, β, γ, δ, etc., amino acids. On washing a column with its adherent amino acid-copper complexes, it is the more acid amino acids that are first removed. No doubt further fractionation could be obtained by this means. The performance of the copper carbonate-alumina column may be indicated by the following data. Depending on the pH of the solution 1 gm. of alumina will absorb 0.92 to 1.5 mg. of α-amino nitrogen in the form of the copper complex at pH 4 and 9 respectively.

Results and discussion

In determining the purity of the isolated γ-aminobutyric acid, several tests were made. Synthetic AB melted sharply at 192 to 193° C uncorr. The isolate from potato tuber melted at 194 to 195° C, and the mixture of synthetic and isolated material melted at 193 to 194° C.

Through the courtesy of the Grasselli Chemicals Department of the Du Pont de Nemours Co., the following ultimate analysis was obtained:

	% C	% H	% O by diff.	% N
AB isolated	46.25	8.49		13.31
	46.26	8.71	31.69	13.39
AB calculated	46.59	8.80	31.03	13.58

Under the standardized conditions for the quantitative ninhydrin determination which have been described (**23, 24**) the isolated AB behaves comparably with the synthetic substance. Samples were dissolved in water and treated on paper with ninhydrin. The blue material was dissolved in 10 ml. of a 1:1 mixture of alcohol and water. The density was read at a path length of 1 cm. at 570 mμ. The optical density for 10 μg. of isolated AB was 0.189 and that for 10 μg. of synthetic AB was 0.184.

It will be recalled that the earlier attempts to isolate AB from potato tuber yielded a product in which there were lines in the x-ray diffraction pattern that were not attributable to AB (fig. 2). Particular interest therefore attaches to the re-investigation of the pure product from the bulk iso-

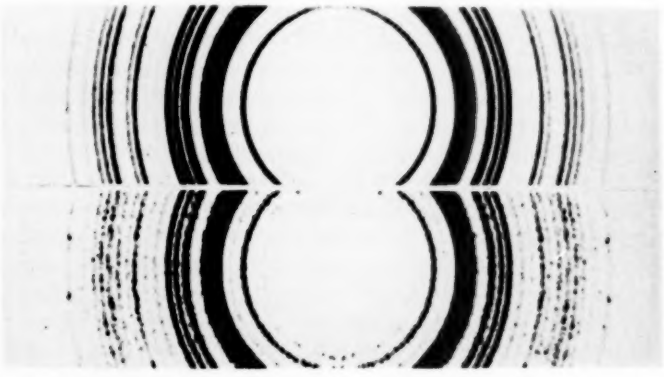


FIG. 3. Above, x-ray diffraction pattern of isolated γ-aminobutyric acid; below, x-ray diffraction pattern of the synthetic material. Standard cylindrical (104 mm. diameter) Powder camera; copper K radiation with Ni filter; time of exposure, three hours.

lation described above by x-ray methods. This work was carried out by Dr. H. Mermagen of the University of Rochester, N. Y., whose results are shown in figure 3. The x-ray diagrams of the synthetic and isolated material are identical. The evidence is conclusive that pure AB has been isolated from from the potato tuber.

After isolation of the crystalline AB from the potato, the mother liquor yielded, on evaporation, a considerable amount (2.2 gm.) of a syrupy material which formed an amorphous glass on removal of water. Presumably this product caused the principal difficulty in the first attempts to prepare pure AB from potato tubers. The full list of tests and determinations made on this substance cannot be given here. At least 50% of the weight of the glass must have been nitrogen free and its nature is still unknown.

Although AB has never been encountered in protein hydrolytic products, it is extraordinarily widely distributed in plants. In fact a plant material in which AB was absent would occasion more surprise in this laboratory than would a further instance of its presence. If alanine, aspartic and glutamic acids are regarded as the amino acids that are most prevalent in plants, then AB clearly falls into the same class. Since its first recognition in the potato tuber (**8**) a very large number of different plant extracts have been examined. In all of these AB was recognized chromatographically.

Evidence for the occurrence in lower plants is fragmentary. Mention has already been made of the isolation of AB from yeast extract (**15**) and to its production by bacteria (**1, 2, 3**). Work has reported its occurrence in *Corynebacterium diphtheriae* (**21**). The occurrence of AB in the fruiting bodies of *Agaricus campestris* and *Ganoderma Tsugae* (on hemlock), in the plasmodium of an unidentified slime mold, in *Chlorella pyrenoidosa* and in *Adiantum pedatum* and *Adiantum cuneatum* has been noted in this laboratory. More extensive observations on the angiosperms have shown that AB is generally present. Table I gives a list of the 28 angiosperms belonging to 14 families and 21 genera in which AB has been recognized. It should be emphasized that in most cases the recognition is chromatographic, *i.e.*, superimposition of synthetic AB with the unknown in two or more solvents by paper chromatography. However, in view of other evidence, there is little reason to doubt that these identifications are correct.

The problems of mode of origin, metabolic role and possible connection of AB with nitrogen metabolism can only be raised in a speculative way. The most obvious presumption is that the AB acid arises by decarboxylation of glutamic acid and reference has been made to the work of SCHALES *et al.* (**16**) who has studied glutamic acid decarboxylase activity in a number of plants. However, there is no apparent correlation between the decarboxylase activity of a tissue and its content of AB. For example, the decarboxylase in carrot root tissue is relatively active, in potato tissue it is relatively inactive which the following data show is no indication of the AB content. In carrot root the glutamic acid decarboxylase activity is 222 mg. CO_2 in 30 mm.^{3}/gm. fresh tissue (**16**) and the AB content is 135 μg./gm. while in potato tuber the glutamic acid decarboxylase activity is 18 mg. CO_2 in 30 mm.^{3}/gm. fresh tissue and the AB content is 300 μg./gm. Admittedly data of this kind do not disprove the possible origin of AB in the manner indicated. Origin in this way, however, would almost certainly demand recarboxylation before the acid could be used in metabolism. Another possible route of synthesis would be by amination of the corresponding keto acid, *i.e.*, succinic acid semi-aldehyde. For this possibility there is, at present, no evidence. If, however, the keto acid could arise as an intermediate of metabolism there would be a possible route to glutamic acid via AB. In other work in this laboratory involving the *in vitro* conversion of α-ketoglutaric acid to glutamic acid, through the oxime or hydrazone, it has been found that glutamic acid is invariably accompanied by γ-aminobutyric acid,

TABLE I

OCCURRENCE OF γ-AMINOBUTYRIC ACID IN ANGIOSPERMS.

Plant	Family	Plant part	Reference
Corn (*Zea mays*)	Gramineae	Fruit	
Rye grass (*Lolium perenne* L.)	Gramineae	Leaf	**21**
Onion (*Allium cepa*)	Liliaceae	Bulb	
Tulip (*Tulipa gesneriana*)	Liliaceae	Leaf and bulb	
Asparagus (*Asparagus officinalis*)	Liliaceae	Shoot	
Daffodil (*Narcissus pseudo-narcissus*)	Amaryllidaceae	Bulb	
Rhubarb (*Rheum rhaponticum*)	Polygonaceae	Petioles	
Beet (*Beta vulgaris*)	Chenopodiaceae	Storage root	**12, 26**
Avocado pear (*Persea americana*)	Lauraceae	Fruit	**13**
Turnip (*Brassica napus*)	Cruciferae	Storage root	
Apple (*Pyrus malus*)	Rosaceae	Fruit and leaf	**13**
Apricot (*Prunus armeniaca*)	Rosaceae	Fruit	**13**
Pear (*Pyrus communis*)	Rosaceae	Fruit	**13**
Prunes (*Prunus domestica*)	Rosaceae	Fruit	**13**
White lupine (*Lupinus albus*)	Leguminosae	Leaf, stem and root	
Snap bean (*Phaseolus vulgaris*)	Leguminosae	Fruit, flower and leaf	
Wax bean (*Phaseolus vulgaris*)	Leguminosae	Fruit	
Lima bean (*Phaseolus limensis*)	Leguminosae	Fruit	
Pea (*Pisum sativum*)	Leguminosae	Fresh and dry seeds	
Carrot (*Daucus carota*)	Umbelliferae	Xylem and phloem of storage root	
Lilac (*Syringa vulgaris*)	Oleaceae	Growing plant and shoot	
Peppermint (*Mentha piperita*)	Labiatae	Leaf, stem and root	
Tobacco (*Nicotiana tabacum*)	Solanaceae	Leaf, root and axillary buds	
Green pepper (*Capsicum frutescens*)	Solanaceae	Pericarp	
Hubbard squash (*Cucurbita maxima*)	Cucurbitaceae	Pericarp and seeds	
Summer squash (*Cucurbita pepo*)	Cucurbitaceae	Fruit	

which arises apparently by decarboxylation of the α-ketoglutarate during the combination of the keto group with the nitrogen compound. If decarboxylation can also occur *in vivo* under similar circumstances it might explain the origin of AB as a by-product of α-ketoglutarate metabolism.

γ-Aminobutyric acid itself does not occur combined in protein. It is important, however, to know whether the nitrogen of the free acid may eventually be incorporated in protein. Since, in the potato, the amides asparagine and glutamine predominate over the AB, conclusive evidence is not easy to obtain. The present status of our evidence on this matter is as follows. When cut potato tissue is exposed to moist air, protein synthesis occurs at the expense of the soluble nitrogen, a fact known since 1901 (**20, 28**). In a given case the protein nitrogen of potato disks increased from 33% of the total to 62% while they were exposed to moist air for a period

of 21 days. Using the quantitative procedures developed in this laboratory (**23, 24**), it was possible to demonstrate that an increase of protein nitrogen from 1.08 to 1.33 mg. N/gm. fresh weight in potato tissue exposed to air was largely at the expense of the amides present, but was nevertheless accompanied by a decrease in AB from 66 to 58 μg./gm. fresh weight.

In other work from this laboratory carrot tissue has been cultured by the aid of a growth factor in coconut milk (**6**) and shown to synthesize protein (**17**). An attempt was made to see whether this tissue would form protein at the expense of AB as the sole source of nitrogen. To this end, a culture solution was used in which nitrate and glycine were replaced by AB, and the growth factor for coconut milk was added as an isolate free of ninhydrin activity. It is true that the tissue grew better in the solution containing the AB than when AB was omitted: the explants weighing initially 3.0 mg. increased to 10.6 $\pm$ 3.2 mg. at the expense only of their own nitrogen compounds whereas with added AB, and in the same period, similar cultures grew to 24.5 $\pm$ 2.5 mg. It was not, however, established that any part of the increase of insoluble nitrogen that occurred was actually at the expense of the nitrogen present in AB. On the contrary it is a feature of these tissue cultures that they contain more AB, though less soluble nitrogen, than the resting tissue from which they were derived. In an experiment designed to test whether Chlorella could use AB as sole source of nitrogen a negative result was obtained. The conclusive evidence to show that nitrogen present originally as AB can, or cannot, appear in protein has still to be obtained.

There are, therefore, still two alternatives. Either AB is an end product of glutamic acid metabolism and its nitrogen is therefore removed from the main stream of metabolism, or else, it is an intermediate through which the nitrogen is continually passing. The ubiquitous presence of AB in plants in small amounts, the relatively greater quantity present in certain growing tissues, its presence even in the growing apex of angiosperms (unpublished results) would all seem to be more consistent with the role of an intermediary.

Summary

The critical evidence for the identification of γ-aminobutyric acid (AB) as a constituent of the potato tuber is reviewed. This evidence is based upon (a) superimposition of synthetic AB upon the naturally occurring substance in phenol : collidine-lutidine chromatograms, (b) the elution of the AB from two-directional chromatograms and the chromatography of a mixture of the isolated and synthetic AB, (c) the proof by use of copper carbonate that the acid in question is not an α-amino acid, (d) the behavior of the ureides of the synthetic and isolated materials.

A new technique for the isolation of AB from the potato tuber is described. Amino acids were separated from non-ionic material on ion exchange columns and then the α-amino acids were separated from AB by their adsorption on a mixed column of copper carbonate and alumina. By this means

pure AB was isolated in good yield of approximately 4 gm. from 13 kilograms of fresh potato tuber. The AB so isolated satisfied tests to show its identity with synthetic AB (melting point, ultimate analysis, x-ray diffraction pattern of the crystals, and quantitative behavior with ninhydrin). The mother liquor from crystallization of AB still contained other substances which again appeared in the form of a glass which did not crystallize. Tests showed that the glass consisted of some residual AB but the rest was nitrogen free and of unknown nature.

The method which has been devised to isolate the AB from potato tuber should be of general application to any case in which it is desired to separate the α-amino acids from the remainder.

The range of plants belonging to 23 genera of flowering plants and 7 genera from other divisions of the plant kingdom and in which AB has been found to occur is described. The substance appears to be ubiquitous in its occurrence in plants. Consideration is given to the metabolic role of AB and its relation to enzyme activity and to protein synthesis.

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AN ATYPICAL GROWTH OF CABBAGE SEEDLING ROOTS. III. TISSUE CULTURE AND PHYSIOLOGICAL COMPARISONS OF TYPICAL AND ATYPICAL ROOTS

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Introduction

Induction of an atypical growth of cabbage roots under sterile conditions (**8**) by *p*-chlorophenoxyacetic acid (POA) has led to a comparative study of typical and atypical growth. The atypical growth (tumor) is a massive tissue, as opposed to the thin, straight morphology of a normal root. The The words tumor or callus as applied to cabbage root growths are intended only as a convenient abbreviation of atypical growth. The material is not malignant, and therefore cannot be considered a true tumor. It differs greatly in degree of organization from the usual plant calluses. It is not as disorganized as the usual callus or tumor tissue culture, but is seen to consist of many abnormal lateral roots growing in a nondivergent, fasciated condition. Since this tissue can be grown under conditions identical with those of the normal roots from which it is derived, it becomes possible to compare the physiological and biochemical characteristics of the two. Aside from the descriptive interest of such a study, the information gathered might be pertinent to the mechanism of induction of the atypical growth, or to the causal relationships between the new biochemical activities and the new morphology.

The purpose of the present work has been to compare tumorous roots of POA-treated plants with typical roots of untreated plants in regard to growth in tissue culture, dry weight, total nitrogen and protein content, and some respiratory characteristics; and to compare the distribution of nitrogen and dry weight in entire treated and untreated seedlings.

Materials and methods

In the experiments involving growth of intact seedlings, cabbage seeds (var. Copenhagen Market) were germinated sterilely and grown on agar media in the dark as previously described (**8**). All chemicals were supplied to the roots of the plants by incorporation in the agar media. After a 14-day growth period, dry-weight determinations of roots and tops of cabbage seedlings were made on the pooled parts of all plants grown in the same medium. The roots and tops were separated, wet weights and lengths were determined, and the plants were then dried at 100° C for 24 hours.

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Total nitrogen was determined according to the micro-Kjeldahl procedure of MA and ZUAZAGA (**10**). Soluble and insoluble nitrogen were determined following a scale modification of the extraction procedures of VICKERY *et al.* (**22**).

Respiration studies were carried out in the Warburg apparatus. Root systems were removed from the agar in which they had been growing, were rinsed vigorously to remove adhering bits of agar, and then placed whole in the Warburg flasks. Following the respiration run, the same tissues were digested and the amount of nitrogen determined. Respiration of three to eight tumors, or of five to 25 roots, was measured in each flask, and rates were calculated per mg. of tissue nitrogen. The usual respiration medium was 2 ml. of M/100 phosphate buffer at pH 5.0, plus 2% sucrose. Carbon dioxide was measured by the direct method of Warburg (**21**).

In tissue culture experiments with excised root material, the previous basal medium was used (**8**) although in liquid form. Nutrient salts, 2% sucrose, and a Neurospora complete vitamin mixture were provided. All growth was carried out in 5 ml. of liquid in a 50-ml. flask, kept on a shaker for the duration of the growth period. A preliminary experiment had shown that both shaking and a small volume of liquid were necessary in order to insure adequate aeration of the tumors. Material for tissue culture was obtained by excision of large portions of tumorous root systems that had been grown on POA plus yeast extract for two weeks. The original and final wet weight of each piece of tissue was determined for each passage. Transfers of material weighing 20 to 50 mg. were made at two-week intervals, under sterile conditions.

Results

TISSUE CULTURE EXPERIMENTS

Cabbage tumors were grown in sterile culture for eight passages of two weeks each; and at the end of that time some tissue masses were increasing their wet weight at a rate of close to five times during a two-week growth period. Figures listed in table I are for the ratio of final to original wet weight of the tissues during two-week passages in 5 ml. of the media indicated. Previous transfers of all material were on POA and yeast extract only (transfers 1 to 3). The tissues grown minus POA were derived from

TABLE I

GROWTH IN TISSUE CULTURE OF TUMOR MASSES AND OF ROOTS DERIVED FROM THEM.

Medium	+POA (tumors) in transfers					−POA (roots) in transfers		
	4	5	6	7	8	4	5	6
Basal	3.6	4.6	2.8			4.2	3.5	1.3
Basal yeast extract	4.0	4.6	5.0	2.9	4.6	9.1	2.1	1.5
Basal proline	5.4	3.7	2.2			4.2	3.7	0.8

passage 3 of the tumor material. Proline was at 9×10^{-4} M, yeast extract is at 1 mg./ml., and POA is at 8×10^{-7} M. Each figure is the average of 7 to 15 tumors. Apparently the tissue might grow indefinitely if handled properly. Dr. L. G. Nickell, formerly at the Brooklyn Botanical Gardens (personal communication) has maintained similarly induced tissues of clover for over 20 months in sterile culture.

Tumor tissue was originally grown on an agar medium. It was found however that tumors retained their positive geotropism and became embedded in the agar as they grew which made accurate and sterile weighing of the tissues very difficult, and led to the adoption of a liquid medium for the passages demonstrating continued growth. In culture, excised cabbage tumors grow at the outer edges only. The regions behind the growing edges remain static, and eventually turn brown and die. Restricted meristematic areas and a rapid rate of enlargement of the cells laid down were seen in the histological preparations (**8**), and explain this gross growth characteristic. The method of growth has some disadvantages for tissue culture work, since it is difficult to obtain equally rapidly growing masses of tissue in replicate. Furthermore, clonal material cannot be obtained in large quantity.

To maintain the atypical form of growth POA was required. If tumorous masses were taken out of the medium with POA, and put in a medium lacking this growth substance, then most of the new growth of the tissue took the form of morphologically distinct roots (fig. 1 A, below). A triple

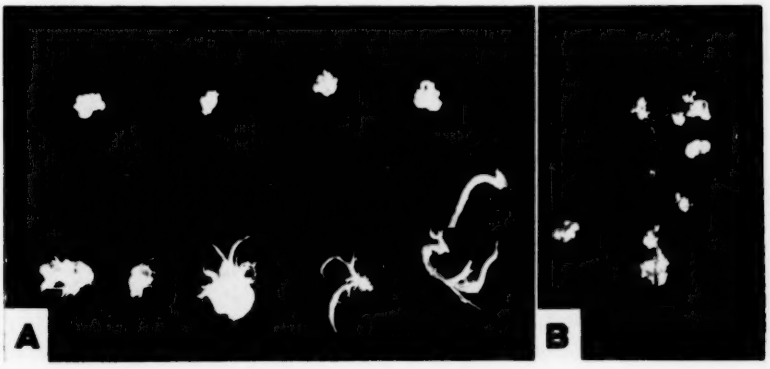

FIG. 1. A. Appearance of cabbage root tumors growing in sterile culture after six passages (top row) and similar material demonstrating new growth in the form of roots (bottom row) after one passage in the absence of POA. The size difference between the two rows is irrelevant, and is due to the fact that the material chosen to be photographed was taken from contaminated cultures that were about to be discarded. Growth occurred in basal medium, with the addition of yeast extract, 1 mg./ml. and POA. ×1.7. B. Cabbage root material, grown alternately in the presence and absence of POA and yeast extract. The tumor mass at top was derived from an original seedling root, the roots radiating out from the tumor grew in the passage minus POA, and the tumors at the ends of these new roots developed when the whole system was put back on POA and yeast extract. ×1.0.

reversal of growth form was found possible, and is illustrated in fig. 1 B. The upper tumor mass originated when a normal primary root was put in the presence of POA. The roots spreading from it grew during a subsequent growth passage in the absence of POA. Then when the whole system of tumor plus new roots was returned to a medium containing POA, massive proliferations again took place in the younger regions of the roots. These experiments demonstrate the impermanence of the changes induced by POA in cabbage seedling roots. They also suggest that root initials are actually present in the atypical growths, and that these require only removal of POA to grow rapidly, normally and individually. This conclusion is supported by the histological evidence of the presence of normal root initials in the atypical tissue (**8**).

In contrast to the tumors, roots derived from excised tumors by removal of POA rapidly die off in culture. Thus in table I, such roots derived after

TABLE II

GROWTH IN CULTURE OF EXCISED CABBAGE ROOTS AND OF TUMORS DERIVED FROM THEM.

Medium	Type of measurement	Passage Number							
		1	2	3	4	5	6	7	8
Basal	cm./root	0.4	0.2	0.0					
Basal plus asparagine	cm./root	1.0	0.7	0.0					
	no. of roots	15	8	0					
Basal plus yeast extract	cm./root	7.3	2.7	3.2	1.6	1.1	0.0		
	no. of roots	19	14	16	6	3	0		
Basal plus POA plus yeast extract	final/original wet wt.					(–)	1.9	2.4	1.1
Basal plus POA plus asparagine	final/original wet wt.							2.1	1.1
Basal plus POA	final/original wet wt.							1.6	0.0

the third tumor transfer were dead by the sixth passage, whereas the original tumors were continuing to grow in the presence of POA. Root tips freshly excised from cabbage seedlings similarly failed to grow for as long a period of time as the excised tumors. These roots would grow for essentially three passages if given yeast extract (although a few exceptional roots lasted for five passages). Without yeast extract they did not grow after the first passage. The growth of these roots is listed in table II. Growth of roots (in media without POA) is listed as centimeter increment per root per two-week transfer period; underneath is listed the number of roots growing in that passage. Growth of the tumors is listed as the final weight divided by the original weight. The tumors (plus POA) were derived from the mature, non-growing regions of roots on basal plus yeast extract, left from transfer 4; and the tumors on POA without yeast extract were derived from passage six of tumors plus yeast extract. Asparagine was at 5×10^{-4} M, yeast extract at 1 mg./ml., POA at 8×10^{-7} M.

As a further demonstration of the better growth of tumors than of the roots from which they are derived, the apparently non-growing regions in the mature part of roots showing some growth in passage 4 (table II) were transferred to a medium containing POA. Despite the fact that only half the root tips without POA survived in the next (fifth) passage, and none in the sixth, the rear areas of these roots did produce tumors under the influence of POA, and these kept growing at an appreciable rate through passages five, six, and seven. Possibly if the tumors had been derived from healthy seedling roots at the same time as the original root tips, they would have kept on growing even after the seventh passage.

Isolated cabbage seedling roots have also failed to grow for more than one transfer in media supplemented with yeast extract plus indoleacetic acid or POA in the range 10^{-8} to 10^{-11} M (**6**). In all experiments the usual vitamins were abundantly supplied, so that these could not have been limiting the growth of cabbage roots.

Nitrogen and dry weight distribution

A greater amount of nitrogen and dry matter is found in plants whose root systems are tumorous, than in normal seedlings (tables III and IV). The formation of root tumors therefore represents a net growth stimulation. All of this excess of nitrogen and dry matter is found in the root systems, which therefore have a much higher proportion of these constituents than usual. Indeed, the tops seem to have slightly less of both than is usual for the typical seedlings. Since the seedlings are grown in the dark, all materials for growth are derived from the medium and must pass through the roots first. It is evident, therefore, that the tumorous root systems must retain a much larger proportion of nutrients than usual, and may even prevent some of the usual amount from reaching the top. The term Qc in tables

TABLE III

DRY WEIGHT DISTRIBUTION IN TYPICAL AND TUMOROUS SEEDLINGS. FIGURES REPRESENT VALUES PER SEEDLING, AVERAGE OF 100 SEEDLINGS FOR EACH FIGURE. NITROGEN IS AMMONIUM CHLORIDE AT 7×10^{-4} M, POA IS AT 8×10^{-9} M, AND SUCCINATE BUFFER AT PH 6.5, AT 8×10^{-3} M. GROWTH PERIOD WAS 14 DAYS.

Growth medium	Root Qc	Root	Top	Entire seedling	
		Dry weight per seedling	Dry weight per seedling	Dry weight per seedling	Dry weight in root
	mg./cm.	*mg.*	*mg.*	*mg.*	%
Basal plus N	1.6	1.23	4.07	5.30	23
Basal plus N plus succinate buffer	1.5	1.24	4.13	5.37	23
Basal plus N plus POA	24.6	4.93	3.88	8.81	56
Basal plus N plus POA plus succinate buffer	4.7	1.72	4.34	6.06	28

TABLE IV

NITROGEN DISTRIBUTION IN TYPICAL AND TUMOROUS SEEDLINGS. FIGURES LISTED ARE THE AVERAGE PER SEEDLING OF 30 SEEDLINGS IN EACH TREATMENT. N IS AMMONIUM CHLORIDE AT 6×10^{-4} M, POA IS AT 8×10^{-7} M, HIGH POA IS AT 2×10^{-6} M. THE GROWTH PERIOD WAS 14 DAYS.

Growth medium	Root Qc	Root	Top	Entire seedling	
		Nitrogen per seedling	Nitrogen per seedling	Nitrogen per seedling	Nitrogen in roots
	mg./cm.	*mg.*	*mg.*	*mg.*	%
Basal	1.4	.03	.189	.219	14
Basal plus POA	6.8	.055	.161	.216	25
Basal plus high POA	18.7	.117	.125	.242	48
Basal plus N	1.6	.022	.170	.192	11
Basal plus N plus POA	18.2	.110	.172	.282	39
Basal plus N plus high POA	30.8	.177	.077	.254	70

III, IV, V, VI is the quotient of wet weight per unit length of the root systems and is a measure of the degree to which a root system is unlike a typical root system, and essentially indicates net thickness. Qc of over 3 indicates some atypical growth; over 10 it indicates strong tumor formation.

An apparent anomaly is found in the lower nitrogen uptake of plants grown in the presence of extra ammonium chloride. Very extensive data from this particular experiment (not shown here) revealed that plants exposed to ammonium chloride, in the absence of POA or buffer, were in a somewhat toxic state. This condition is reflected in the lower nitrogen uptake.

Nitrogen as a mobilizing factor

Several experiments were performed to determine the movement of dry matter and nitrogen within the cabbage seedling as a consequence of tumor formation in the roots. The results from one such experiment lasting 14 days, are listed in table V. Values with urea in table V should be compared with those at pH 6, since the medium was maintained at or above this pH by the presence of urea. The inability of low pH to duplicate the effects of the nitrogen sources and the effect of nitrogen in the medium to reduce the amount of nitrogen going into the tops should be noted. A net gain in dry weight and nitrogen in the roots was observed on all media during the 14-day growth period, and usually in the tops as well. With increasing POA present in the medium, there was a greater gain of nitrogen and dry matter in the roots, but the increment of both of these in the tops was reduced.

The tendency to prevent nitrogen or dry matter from getting to the tops was enhanced by extra nitrogen present in the medium. In the extreme case (table V, medium containing high POA, or low POA plus ammonium chloride) there was a net translocation of nitrogen and dry matter out of the

TABLE V

NITROGEN AND DRY WEIGHT INCREMENTS OF CABBAGE SEEDLINGS GROWN IN THE PRESENCE OF POA. FIGURES ARE THE AVERAGE PER SEEDLING OF 25 SEEDLINGS EACH. POA IS AT 8×10^{-7} M, HIGH POA IS AT 2×10^{-6} M, UREA AND AMMONIUM CHLORIDE ARE AT 8×10^{-4} M. CONTROLLED PH OF 5 AND 6 ARE MAINTAINED BY SUCCINATE BUFFER AT 4×10^{-3} M. GROWTH PERIOD WAS 14 DAYS.

Growth medium	Qc	Dry weight increment			Nitrogen increment		
		Root	Top	Seedling	Root	Top	Seedling
		mg.	mg.	mg.	mg.	mg.	mg.
Basal plus POA	10	2.3	0.9	3.2	.055	.029	.084
Basal plus high POA	23	2.9	0.0	3.0	.116	−.010	.106
Basal plus POA plus NH^4Cl	23	4.2	−0.4	3.8	.148	−.012	.136
Basal plus POA plus urea	16	3.0	0.2	3.2	.099	.018	.117
Basal plus POA pH 5	13	2.8	0.7	3.5	.109	.016	.125
Basal plus POA pH 6	5	1.5	1.2	2.7	.025	.047	.072

tops and into the roots. This effect was most clearly seen at intermediate times during the growth period, in this and other experiments. Thus the presence of extra nitrogen in the medium together with POA is seen to assist in the mobilization of nitrogen and dry weight to the growing tumorous root system. That the effect is not due to manipulation of the pH of the medium is seen in table V, where a low pH maintained by a non-nitrogenous buffer could not duplicate the effects of extra ammonium and a nitrogen source that causes the pH of the medium to rise (*i.e.*, urea) is also seen to assist in the translocation of materials to the roots, or at least in preventing their translocation to the tops.

The amount of insoluble (protein) nitrogen was determined for typical and atypical root systems. Seedling root systems that were tumorous, and normal roots grown in media lacking POA, showed a comparable per cent. of nitrogen in insoluble form (74 to 78%). Root systems growing on basal medium plus ammonium chloride by itself, however, at times seemed to have a slightly lower per cent. of protein, probably attributable to their toxic conditions. Since there is a larger amount of nitrogen in tumorous roots than in typical roots, the total protein formed is greater in tumorous root systems.

Respiration experiments

Oxygen uptake, carbon dioxide output, and R.Q. (ratio of CO_2 output to O_2 uptake) of roots were compared with those of tumors in a number of experiments. All the essential points are illustrated with material obtained at different times during the growth period of one experiment (table VI). The respiration of roots varied between 100 and 200 microliters of O_2 uptake per mg. N per hour. The rate of respiration of tumors is of the same order of magnitude on a mg. N basis. Roots removed at later times from the

TABLE VI

RESPIRATION RATES OF ROOTS AND TUMORS GROWN ON THE MEDIA SHOWN FOR VARIOUS LENGTHS OF TIME. B STANDS FOR BASAL MEDIUM, N IS AMMONIUM CHLORIDE AT 8×10^{-4} M, P IS POA AT 8×10^{-7} M, PP IS POA AT 2×10^{-6} M.

Parts	Medium	Final Qc	Q_{O_2}* at day						R.Q.** at day					
			0	2	5	10	13	16	0	2	5	10	13	16
Roots	B	1.4	189	....	....	....	....	104	1.02	....	....	....	....	1.05
	BN	1.6	....	180	196	124	83	72	....	1.04	1.06	1.00	1.05	1.12
Tumors	BPP	19.0	177	....	....	....	115	....	1.00	....	....	....	1.27	....
	BNP	19.0	....	176	192	124	136	144	....	1.09	1.08	1.16	1.12	1.15
	BNPP	31.0	....	....	....	....	85	....	....	....	....	1.26	1.62	....

* In microlitres O_2/mg. tissue N/hour.
** Ratio of CO_2 given off/O_2 taken up.

growth medium had decreasing rates of respiration after the fifth day. The most marked decrease was associated with normal roots in a toxic condition, growing in unbuffered ammonium chloride plus the basal medium.

The R.Q. of respiring roots was almost always close to 1.0. However, tumor respiration is characterized by a consistently higher R.Q. than that found for roots. All tumors with Qc (index of tumor morphology) greater than 5 mg./cm. had an R.Q. of 1.12 or more. The highest R.Q. observed for very tumorous root systems was 1.6. The increased R.Q. is a characteristic of the tumorous growth resulting from the application of POA, and is not caused by the direct application of POA. Normal roots that were put in the presence of POA at 2×10^{-6} M, a concentration high enough to cause tumor formation in the absence of extra nitrogen, maintained an R.Q. of 1.0 for at least the first four hours (table VII, 0-day measurements). The rise in R.Q. resulting from growth in the presence of POA could be distinctly observed only on the tenth day, although indications of a rise were obtained as early as the second day.

It would seem from the figures in table VI that POA at 2×10^{-6} M causes a slight decrease in the respiration rate of seedling roots (0-day column). The decrease is not a very large one, however, and has not been repeated enough times to determine whether it is a significant one or not. Any such slight inhibition of respiration is eliminated after further growth in the presence of POA, moreover.

SENSITIVITY TO REDUCED ATMOSPHERIC PRESSURE

When the inhibition of tumor formation by pH was first noticed, it was believed that the inhibition involved oxidative utilization of the organic acid buffers (7). Growth under reduced atmospheric pressure (and therefore reduced oxygen tension) failed to reveal any selective inhibition of the organic acid effects, such as might have been expected if their oxidative utilization were responsible for inhibition of tumor formation. These experi-

ments did show, however, that tumorous growth is considerably more sensitive to a 50% reduction in atmospheric pressure than is the growth of normal roots.

Discussion

The data presented on tumor respiration, on dry matter and nitrogen uptake and distribution, define characteristics of the atypical growth itself, but offer no evidence in regard to any immediate biochemical effects of POA at these concentrations. They all occurred only after a period of growth in contact with POA, and not on direct application of the growth substance itself. Similar secondary effects have been observed in regard to various constituents of many plants treated with phenoxyacetic compounds, especially 2,4-D (**11**). In our experiments, a net growth stimulation occurred, as evidenced by greater total uptake of dry matter and nitrogen from the medium, accumulation of these in the tumors, and greater amounts of protein formation and total material respired. The distinction between tumors and normal roots is emphasized by the fact that this growth stimulation occurs at concentrations of POA which almost completely inhibit normal root elongation (**8**).

Some previous investigations have shown phenoxyacetic compounds (**1, 16**) to restrict the total uptake of salts by treated plants. In those two instances lower uptake was correlated with a reduced root volume. In the present case there was no reduction of root volume, although the absorbing surfaces may have been equal to or even smaller than those of typical root systems. In at least three cases, application of growth substances to the roots of plants has been shown to cause translocation of materials to the roots and accumulation there (**1, 4, 16**). In the present case, again, application of POA to the roots of cabbage seedlings resulted in a net translocation of dry matter and nitrogenous materials from the tops to the roots, especially when higher concentrations of POA were used.

It was of some interest to find that, when POA was present, the process of translocation of nitrogen away from the tops was accelerated by extra nitrogen in the agar medium. This fact is of course explained in a general way by the formation of larger and more vigorous tumors by POA when extra nitrogen is present in the medium (**6**). Mobilization of foodstuffs here cannot be attributed directly to the growth substance but must occur indirectly through the new growth processes which result from the application of growth substance which can be compared, however, with the use of the term mobilizing hormone (**3**, p. 445.)

In previous experiments (**6**) it was found that an excess of nitrogen in the medium increased the activity of all concentrations of POA in the formation and growth of root tumors. The present experiments show that tumorous growth is more rapid than that of normal roots, and more nitrogen is utilized in protein formation. It might therefore be thought that the extra nitrogen is serving only to support the more rapidly growing atypical tissue.

However, the excess nitrogen had the largest effect together with low concentrations of POA, which were then enabled to form small tumors. The growth of small tumors should not have required as large supplies of excess nitrogen as that of large tumors; nevertheless it was seen that stimulation of tumor formation by low concentrations of POA required more extra nitrogen than did that of high concentrations of POA. It may still, therefore, be concluded that extra nitrogen acts as a specific co-factor with POA in the formation of these tumors.

Neither a direct nor an indirect effect of POA on the rate of respiration was observed with this material, although indirect effects with toxic concentrations (**2, 15, 17, 18**) and direct effects with very high (**23**) or very low (**9**) concentrations of 2,4-D have ben noted. The outstanding effect noted with cabbage roots is an increase in R.Q. as they become tumorous under the influence of POA. Again, this is an indirect effect of the growth substance. Of the previous reports of a higher R.Q. following application of 2,4-D (**5, 17, 20**) only one (**20**) clearly seems to be a direct effect, and this was not a very large one.

The tumorous cabbage seedlings were grown in the dark, with sucrose as sole carbon source. Under these conditions the net excess production of carbon dioxide represents a partially fermentative respiration. There should be, therefore, some fermentation products being formed and stored in the cabbage tumors. No attempt has been made, however, to determine the existence or composition of such products. Cabbage tumors seem to grow in an intermediate state of organization. Although not root-like, they are still far from being disorganized callus tissues. The intermediate state is emphasized by retention of positive geotropism, a physiological characteristic of true roots. This phenomenon causes some difficulty in quantitative tissue culture work, as stated in the experimental section. It is interesting to note, in addition, that tumors are growing in concentrations of POA sufficient to inhibit normal root elongation almost completely. Nevertheless, the tumors can still respond to gravity, and presumably to alterations in the distribution of internal supplies of auxin.

The phenoxyacetic series of synthetic growth substances have not yet been used very much as agents for producing or maintaining plant tissues in culture. Steward and Caplin (**19**) have reported that potato tuber tissue could be induced to grow as a callus in culture, in the presence of 2,4-D and coconut milk combined. Potato tissue, like the excised cabbage roots (**24**) previously could not be grown in the absence of 2,4-D. From the work of Steward and Caplin, and the present experiments, it seems likely that many plant materials impossible to culture by methods yet attempted, might be brought into excised culture by the use of substituted phenoxyacetic acids with or without accessory factors.

The better growth of excised tumors than of roots under these conditions is perhaps the most interesting indication of an alteration of the tissue metabolism by POA. Whatever was limiting the growth of detached roots

could either be replaced, supplied or circumvented by the influence of POA. An analogous situation, in which roots derived from carrot callus had a nutritional requirement (thiamine) which was obligately replaced in the parent callus by the growth substance indoleacetic acid, was reported by NOBÉCOURT (**14**).

It would be of considerable interest to find out what factors are necessary for the growth of cabbage root tips, since these would be closely related either to the mode of action of POA directly, or to the new and different biochemical metabolism initiated by POA. It is pertinent to note that the ability to maintain growth *in vitro* is as dependent on the continued presence of POA as are the morphological changes caused by POA.

Summary

Cabbage root tumors induced by *p*-chlorophenoxyacetic acid (POA) can be maintained for at least eight passages of two weeks each in tissue culture. Normal roots, however, die out rapidly in excised culture. The formation of tumors from roots is readily reversible, since the continuing presence of POA is necessary to prevent the atypical growths from reverting to normal growth. Tumors continue to show positive geotropism in culture.

Formation and growth of tumors represents a net growth stimulation. Wet weight, dry weight, total nitrogen, and total protein are all higher in tumorous root systems than in normal root systems. Total uptake of material by the entire seedling is greater when the roots are tumorous than when they are normal. Although the tumorous root systems are accumulating large amounts of materials, accumulation in the tops does not show a corresponding rise. In the most extreme cases of tumorization, nitrogen and dry materials may be drained out of the tops and into the roots.

Tumors are characterized by a respiratory quotient greater than unity, whereas the R.Q. of normal roots is always close to 1.0. The rise in R.Q. is not an immediate effect of adding POA, but only occurs after a few days' growth in the presence of the growth substance.

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SOME RELATIONSHIPS OF STEROLS TO PLANT GROWTH

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Introduction

In spite of the universal occurrence of steroids in plants, the role of these materials remains quite unknown. That steroids possess physiological activity for the higher plant was in fact demonstrated only after the discovery of estrogenic principles in plants in 1926–1927 by LOEWE and SPOHR (**22**), DOHRN *et al.* (**12**), and LOEWE *et al.* (**20, 21**).

The status of our knowledge of the influence of the animal steroid hormones on the vegetative growth and reproductive development of plants has been extensively reviewed by LÖVE and LÖVE (**23**). There appear to be several well established examples of the influence of estrogenic materials on vegetative growth. Effects of flowering appear to be generally secondary to this effect. HYMLÖ (**17**) has, however, demonstrated an influence of estrone and of testosterone on sexual expression in spinach, and LÖVE and LÖVE (**23**) have been able to suppress either anther or gynecium growth in Melandrium by application of estrone or testosterone respectively.

In addition to the effects which estrone exerts when applied directly to intact plants, there are additional instances of effects of estrone on growth of isolated plant parts or tissues. Such effects have been observed by KÖGL and HAAGEN-SMIT (**18**) and by BONNER and AXTMAN (**10**) with isolated pea embryos, by FIEDLER (**14**) with isolated root tips of corn, by WEYLAND (**25**) with *Allium cepa* roots, and by GIOELLI (**15**) with cultures of carrot root. In all of these instances additions of estrone to the culture medium have served to cause a general increase in growth rate. That the synthetic estrogen, diethylstilbesterol, can replace estrone in promotion of plant growth has been established by ZOLLIKOFER (**26**).

The effects of testosterone treatment of higher plants, other than the influence of sex expression noted above, have been quite varied. VON EULER (**13**), for example, found that inhibition of seedling development resulted when seeds of *Lepidium sativum* or *Hordeum vulgare* were treated with testosterone at concentrations of 0.01% as well as with estrone. Similarly, GLASER and RANFTL (**16**) found testosterone harmful to growth of hyacinth bulbs while DAVID and GOUZON (**11**) found testosterone to be without effect on the species studied by them.

Certain microörganisms are influenced by additions of steroids to the nutrient medium. The *Rhodotorula sugari* and *R. glutinis*, two of several yeasts investigated by WEBER (**24**), responded (particularly in the presence of Zn) with increased growth to applications of estrone, estradiol benzoate,

or testosterone. NÉKÁM and POLGÁR (**19**) found growth retardation of *Trichophyton crateriform* and *Staphylococcus aureus* by estrone, and growth arrest by either androsterone or testosterone.

No function nor physiological activity has as yet been found for the universally distributed phytosterols and mycosterols so far as plants are concerned although these substances have of course found extensive use in the synthesis of products which have hormone or vitamin activity in higher animals.

The saponins constitute a group of steroids of wide but sporadic occurrence in the plant kingdom. BALANSARD and PELLESSIER (**1, 2, 3, 4, 5, 6, 7**) in a series of papers published between 1943 and 1945 have shown that saponins include substances capable of eliciting plant growth responses. These authors used various concentrations (10 to 1000 μg. per ml.) of quillaja, polygala, saponaria, and sapindus saponins. The growth rate of isolated wheat embryos was approximately doubled by optimal concentrations of saponin, although higher concentrations were toxic. Saponin applications also brought about increased rate of development of shoots and roots by begonia leaves. The treatment of seeds of cereals and tomato with dilute solutions of saponins accelerated germination and increased subsequent growth rate. The application of dilute saponin solutions to tomato roots resulted, however, in growth inhibitions, preceded in some instances by a short period of increased growth. Seeds of peas absorbed water more rapidly in the presence of saponins. BALANSARD *et al.* (**8**) found similar effects of saponin on water absorption by seeds of corn, with a subsequent more rapid germination. VON EULER (**13**) germinated seeds of *Lepidium sativum* and *Hordeum vulgare* in the presence of digitonin, quillaja saponin, or sodium cholate but found only growth inhibitions due apparently to the use of excessively high concentrations.

In the present work, a survey of the physiological activity of a wide variety of steroids is presented. The comparison is based on effects on the growth of isolated pea embryos in aseptic culture, according to the method of KÖGL and HAAGEN-SMIT (**18**) and BONNER and AXTMAN (**10**).

Materials and methods

All of the experiments were conducted with excised embryos of pea, variety Alaska. The methods of sterilization and culture were identical with those of BONNER and AXTMAN (**10**). The culture medium was that prescribed by BONNER and ADDICOTT (**9**) for growth of pea roots, except that the agar medium contained 2% rather than 4% sucrose and the culture vessels were 75 × 250 mm. Pyrex test tubes. The basal medium contained $Ca(NO_3)_2 \cdot 4\,H_2O$ 170 mg./l.; $MgSO_4 \cdot 7\,H_2O$ 21 mg./l.; KNO_3 85 mg./l.; KCl 61 mg./l.; KH_2PO_4 20 mg./l.; ferric tartrate 1.5 mg./l.; agar 1%; sucrose 2%. The plants were grown at 25° C in the dark. Approximate measurements of stem growth were made weekly for three weeks, and after four weeks the plants were removed from the culture tubes for measurement

TABLE I

AVERAGE GROWTH OF 11 CONTROL SERIES OF EXCISED PEA EMBRYOS IN UNSUPPLEMENTED BASAL MEDIUM. GROWTH IN DARK AT 25° C. INITIAL LENGTH OF STEM, APPROXIMATELY 2 MM.

Stem length				Root length after 4 weeks
After 1 week	After 2 weeks	After 3 weeks	After 4 weeks	
mm.	*mm.*	*mm.*	*mm.*	*mm.*
17.7 ± 2.4	32.1 ± 4.5	43.3 ± 5.1	56.0 ± 9.0	56.9 ± 9.9

of final stem and root lengths. The individual stems and roots of each group were then separated for dry-weight measurements. Twenty embryos were cultured and grown in each experiment for each treatment. The number of plants finally harvested in each group was approximately 18 since an occasional embryo either failed to develop or formed adventitious roots or shoots due to injury at excision. These individuals were discarded.

Accessory substances were added to the culture medium in water, ether, or alcohol solutions, and controls were prepared using comparative amounts of pure solvents. The addition of ether was without significant effect. Alcohol, however, tended to increase growth somewhat after two weeks. Several compounds, principally the esterified compounds, precipitated from the cold medium, and hence concentrations were limited in these cases by a solubility factor.

Results

The results, as recorded in the tables, are given as per cent. of the growth of the control. Additional notations indicate statistical significance of growth in the individual treatments from growth in the unsupplemented basal medium. The growth of the individual control series was quite uniform as between successive experiments. Table I presents the average growth measurements for 11 control groups.

TABLE II

GROWTH RESPONSES OF EXCISED PEA EMBRYOS TO VARIED ACCESSORY FACTORS. MEASUREMENTS AFTER FOUR WEEKS.

Accessory substance	Concentration	Growth in length as per cent. of control	
		Stem	Root
	mg./l.		
Vitamin B_1	1	93	133*
Vitamin B_6	1	81*	89
Biotin	0.1	10**	7**
Biotin	0.01	94	110
Biotin	0.001	103	102
Casein hydrolyzate	100	89	121*

*Significant at 5% level.
**Significant at 1% level.

In preliminary experiments several groups of embryos were cultured in the presence of nonsteroidal accessory substances in order to arrive at as optimal a medium as possible for this variety of pea. The results of these experiments are given in table II. Vitamin B_1, at 1.0 mg./l., and casein hydrolysate, 100 mg./l., increased root growth significantly. Nicotinic acid, pantothenic acid, ascorbic acid, and adenine were substantially inactive. Vitamin B_6, at 1.0 mg./l., inhibited shoot growth, and biotin above 0.01 mg./l. was inhibitory either alone or in the presence of the B vitamins mentioned above. Accordingly, none of these accessory substances was used in the culture medium.

TABLE III

GROWTH RESPONSES OF EXCISED PEA EMBRYOS TO STEROID ANIMAL HORMONES. MEASUREMENTS AFTER FOUR WEEKS.

Accessory substance	Concentration	Growth in length as per cent. of control		Dry weight as per cent. of control	
		Stem	Root	Stem	Root
	mg./l.				
Estrone	10.0	102	108	93	99
	1.0	120*	115	118	114
	0.1	104	96	107	99
	0.01	100	106	106	125
Testosterone propionate	10.0	35**	90	56	77
	1.0	75**	85	89	83
	0.1	75**	90	84	93
	0.01	104	104	106	103
Dihydroxy estrin	10.0	87	94	83	89
	1.0	90	85	83	105
Methyl-androstenediol	1.0	83	81	97	107
Cortisone	10.0	15**	25**	....	....
	1.0	78**	112	92	116

*Significant at 5% level.
**Significant at 1% level.

Estrone, in accordance with the earlier findings, exhibited considerable activity in increasing growth of pea embryos, increasing stem growth by a maximum of 20% at its optimum concentration of 1 mg./l. Testosterone on the contrary was highly inhibitory, exerting a growth-depressing effect which disappeared only at concentrations of 0.01 mg./l. and below. Two other compounds of the animal sex hormone series, dihydroxyestrin and methyl androstenediol, were also slightly inhibitory and, in common with others of the steroids tested, showed inhibitory action to an extent which approached statistical significance. Cortisone, in concentrations of 1 and 10 mg./l. greatly inhibited shoot growth although only the higher concentration inhibited growth of the root.

All of the other typically animal sterols summarized in table IV were ineffective or inhibitory to growth of pea embryos at all concentrations

TABLE IV

GROWTH RESPONSES OF EXCISED PEA EMBRYOS TO CHOLESTEROL AND RELATED ANIMAL STEROLS. MEASUREMENTS AFTER FOUR WEEKS.

Accessory substance	Concentration	Growth in length as per cent. of control		Dry weight as per cent. of control	
		Stem	Root	Stem	Root
	mg./l.				
Cholesterol	10.0	93	87	101	88
	1.0	100	108	98	106
	0.1	90	80**	88	87
	0.01	85	87	96	99
Cholic acid	0.1	78*	80**	83	82
	0.01	76*	86	90	93
Desoxycholic acid	0.1	96	90*	91	97
	0.01	82	88	83	89
α-Hyodesoxycholic acid	10.0	69**	68**	82	70
	1.0	104	80*	106	88

*Significant at 5% level.
**Significant at 1% level.

tested. Thus cholesterol, from two different sources, caused small and somewhat irregular inhibition, particularly of root growth. Cholic acid inhibited both shoot and root growth at low concentration, desoxycholic acid was less effective, and α-hyodesoxycholic acid was inhibitory only at relatively high concentrations.

TABLE V

GROWTH RESPONSES OF EXCISED PEA EMBRYOS TO SAPONINS. MEASUREMENTS AFTER FOUR WEEKS.

Accessory substance	Concentration	Growth in length as per cent. of control		Dry weight as per cent. of control	
		Stem	Root	Stem	Root
	mg./l.				
Saponin, Merck Sample 1	50.0	92	115	107	110
	20.0	98	104	103	103
	10.0	124*	100	118	105
	10.0	126*	111	107	114
	1.0	106	102	102	96
	1.0	115	106	105	117
Saponin, Merck Sample 2	10.0	140**	143**	120	125
	1.0	119*	91	105	105
Saponin, Penick	10.0	117*	98	104	104
	1.0	134**	132*	117	124
Diosgenin saponin	10.0	143**	147**	120	136
	1.0	113	123	110	135

*Significant at 5% level.
**Significant at 1% level.

The steroids most active in promotion of growth of pea embryos have been found among the saponins. The present results summarized in table V roughly parallel those of Balansard and Pellessier in which the rate of growth of wheat embryos was nearly doubled by saponin application. In contrast to the work with wheat, however, no growth-inhibition by saponins was noted with pea embryos, even in concentrations as high 50 mg./l. Optimum growth of stems occurs in all cases near 10 mg./l. Root response in general followed the response in stem growth.

TABLE VI

GROWTH RESPONSES OF EXCISED PEA EMBRYOS TO TYPICAL PLANT STEROLS. MEASUREMENTS AFTER FOUR WEEKS.

Accessory substance	Concentration	Growth in length as per cent. of control		Dry weight as per cent. of control	
		Stem	Root	Stem	Root
	mg./l.				
Ergosterol	10.0	89	72*	82	86
	1.0	91	94	89	83
Sitosterol	10.0	79	111	86	91
	1.0	87	102	85	90
Diosgenin acetate	10.0	102	98	106	95
	1.0	82*	96	93	91
Stigmasterol acetate	10.0	90	110	100	103
	1.0	96	96	101	98
Stigmasterol hydrate	10.0	94	93	94	94
	1.0	85	91	85	94
Hecogenin acetate	10.0	96	104	98	98
	1.0	96	102	100	100
$\Delta^{9,11}$-Hecogenin acetate	10.0	91	94	88	90
	1.0	80	85	84	82
Dehydrodiosgenin acetate	10.0	104	104	100	98
	1.0	98	116	97	106

*Significant at 5% level.

The saponin preparations used included three commercial samples of relatively crude material containing mixtures of materials as well as one chemically defined compound, diosogenin saponin. The latter was fully as effective in promoting pea embryo growth as the crude materials.

The remaining compounds tested for effectiveness on the growth of pea embryos were plant products, naturally occurring or synthetically modified. These included a sitosterol preparation, stigmasterol hydrate and acetate, diosgenin acetate, dehydrodiosgenin acetate, hecogenin acetate, $\Delta^{9,11}$-hecogenin acetate and ergosterol. Results with these materials are summarized in table VI. All were either ineffective or slightly inhibitory over the concentration ranges tested.

Discussion

It has been shown that the promotion of pea embryo growth by sterols and related materials possesses a definite chemical specificity. Of all the sterols and sterol derivatives tested in the present investigation, only one, estrone, caused significant increases in growth. This material has not only been previously shown to be effective in the pea embryo test but to influence the growth of other higher plant tissues and species as well. It is particularly noteworthy that the well known plant sterols ergosterol, stigmasterol, and sitosterol were found to be without growth-promoting effect.

That the growth-promotion of pea embryos by estrone is attended by a marked chemical specificity is stressed by the fact that the closely related dihydroxyestrin was found to be totally ineffective. In addition, the growth-promotion of pea embryos by estrone finds its counterpart in the relatively specific growth-inhibition exerted by testosterone. This material in concentrations as low as 0.1 mg./l., was found to bring about significant reduction in embryo growth. Growth-reductions of a less striking nature were caused by a variety of other steroidal materials. It is nevertheless striking that the most effective growth-inhibitor in these experiments is testosterone just as estrone is the most effective growth-promoter.

The steroidal saponins constitute another group of substances with marked growth-promoting effects on cultured pea embryos. That growth-promotion by saponins is not due to their possible content of estrone is indicated by the fact that in one instance, that of diosgenin saponin, a presumably pure material was found to bring about growth-promotion. It is still quite uncertain, however, whether promotion of growth is actually exerted through a mechanism similar to that affected by the sterols proper. It might, for example, be possible that saponins with their marked surface properties affect in some unknown manner the wetting or other relations to water of embryos and seedlings in culture. Still, the growth-promotions brought about in these experiments by the steroidal saponins are striking and deserve further chemical attention.

Do the growth-promotions and growth-inhibitions by sterols and steroidal materials described in this paper and in earlier work have any relation to the normal growth of the plant? This question cannot be answered since such elementary facts as whether or not sterols and steroidal materials are transported within the plant so that they might then act in hormonal capacities are not known. Neither is there any insight into the biochemical role of sterols and steroidal materials. That sterols may indeed play significant physiological roles is, however, suggested by the occurrence of estrogenic substances in plant material coupled with the fact that such estrogenic substances as estrone can be shown as in the present case to possess specific physiological activity for higher plants.

Summary

The embryos of mature pea seeds respond by increased growth when they are supplied in aseptic culture with certain sterols or steroidal materials.

Of the seventeen sterols and steroids tested, only one, estrone, possessed the activity of significantly increasing growth of pea embryos in culture. Other compounds and in particular, testosterone, were highly effective in inhibition of growth. Naturally occurring plant sterols such as ergosterol, stigmasterol, and sitosterol were without effect on pea embryo growth. The steroidal saponins both in crude preparation and in one instance in chemically pure form exert marked effects in increasing the growth of excised pea embryos.

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THE EFFECT OF SODIUM TRICHLOROACETATE UPON THE METABOLISM OF WHEAT SEEDLINGS (*TRITICUM VULGARE* L.)[1]

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Introduction

Recent studies (**1, 3, 4**) have demonstrated that the salts of trichloroacetic acid (TCA) are soil sterilants and are, therefore, useful as herbicides for the control of annual and perennial weedy grasses. Since little information (**1, 5**) is available on the physiological action of TCA on plants, a study was made to determine its effect upon the various chemical constituents of wheat seedlings. This paper presents data on respiratory activity and chemical analyses for amino acids, carbohydrates, and ether extract for the shoots and roots of wheat seedlings grown in soil treated with sodium trichloroacetate and non-treated soil.

Materials and methods

Seeds of spring wheat (var. Henry) were selected for uniformity of size and planted in sandy loam soil, contained in 40 flats. Each flat contained 400 plants, and two replications of 2000 plants each, grown in treated and non-treated soil, were used to obtain the material for analyses. Application of TCA (formulated as the water-soluble sodium salt of trichloroacetic acid and supplied by the Dow Chemical Company of Midland, Michigan) was made by mixing the salt in the soil at a rate of 168 mg. per flat or the equivalent of 30 lbs. per acre prior to sowing of the seeds. The soil pH was adjusted to 6.5 by the addition of lime. The application of TCA did not appreciably change the pH. The soil was kept moist with water for the duration of the experiment and the flats were kept in a greenhouse at temperatures ranging from 70 to 75° F during the day and 65° F at night. After 14 days the plants were harvested, dried, and separated into root and shoot tissue. The material was further dried at 70° C and 5 mm. pressure for 12 hours to constant weight, ground to pass through a 60-mesh sieve and stored in glass jars until analyzed.

Amino acids were determined microbiologically with the organisms *Lactobacillus arabinosus, Streptococcus faecalis,* and *Leuconostoc mesenteroides.* The media used in the various determinations were essentially the same as those described by SAUBERLICH and BAUMANN (**6**). Samples were prepared

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for assay by the method described by STOKES *et al.* (**10**) with the exception of the tryptophan samples which were prepared according to the method of WOOLEY and SEBRELL (**13**). Carbohydrates were determined by the procedure outlined by SELL *et al.* (**7**). The methods of analyses for nitrogen and ether extract were standard procedures (**2**).

For respiration measurements plants were grown in treated and non-treated soil as previously described. Before the measurements were made, the plants were washed in aerated, running tap water for two to three hours. The rate of oxygen uptake was determined separately for shoot and root tissue of each plant in a standard Warburg apparatus in 0.02 M, pH 5.7 phosphate buffer (**9, 11**). The shoots were cut into 5-mm. lengths for the respiration measurements while the roots were placed intact in the Warburg vessels.

Results and discussion

The germination of the seeds was not affected when planted in soil previously treated with TCA. Signs of injury became evident about 10 days after planting when the seedlings developed roots, and the extent of injury increased as the root system became more widespread. The first noticeable symptom is the appearance of a yellow color at the tips of the older leaves. Eventually the entire leaf turns yellow, and the basal part of the leaf thickens with the formation of a dark green color resulting in the death of the seedling. Plants grown in the treated soil show retarded growth and the degree of injury is dependent upon the quantity of TCA applied.

The results of the chemical analyses are summarized in tables I, II, III, and IV. Each entry is the result of a single determination of each replicate.

TABLE I

AMINO ACID AND NITROGEN CONTENT OF SHOOTS FROM WHEAT PLANTS GROWN IN SODIUM TRICHLOROACETATE TREATED AND NON-TREATED SOIL.

	Replicates of sample				Replicates of crude protein			
	Non-treated soil		Treated soil		Non-treated soil		Treated soil	
	1	2	1	2	1	2	1	2
	%	%	%	%	%	%	%	%
Nitrogen	5.39	5.37	6.67	6.65				
Protein (N×6.25)	33.69	33.56	41.69	41.56				
Methionine	.35	.36	.39	.40	1.03	1.07	.94	.96
Lysine	1.43	1.43	1.54	1.54	4.24	4.26	3.75	3.71
Valine	1.92	1.94	1.83	1.82	5.69	5.78	4.39	4.38
Leucine	1.77	1.77	1.83	1.89	5.25	5.27	4.39	4.55
Arginine	1.61	1.62	1.99	2.07	4.78	4.83	4.77	4.98
Histidine	.70	.73	.78	.80	2.08	2.18	1.87	1.92
Phenylalanine	1.21	1.24	1.35	1.33	3.59	3.69	3.24	3.20
Isoleucine	0.68	0.68	0.67	0.67	2.03	2.03	1.61	1.61
Threonine	1.27	1.29	1.27	1.27	3.77	3.84	3.05	3.06
Tryptophan	.59	.58	.40	.36	1.75	1.73	.96	.87

The data in table I expressed as per cent. of amino acid in the sample show the following trends: The shoot tissue of the plants grown in treated soil contained more protein and arginine than that of the controls. The percentage of tryptophan was less in the soil-treated plants. Slight differences were noted for methionine, lysine, valine, leucine, histidine, phenylalanine, and threonine. The percentage of isoleucine did not change. Expressing the data in table I as per cent. of amino acids in the crude protein, a somewhat different pattern was observed. The shoot tissue of the soil-treated plants contained slightly less lysine, valine, leucine, histidine, phenylalanine, isoleucine, threonine, and tryptophan than did the controls. Approximately the same percentage of methionine and arginine was observed in both the plants grown in treated and non-treated soil. The data presented on the

TABLE II

AMINO ACID AND NITROGEN CONTENT OF ROOTS FROM WHEAT PLANTS GROWN IN SODIUM TRICHLOROACETATE TREATED AND NON-TREATED SOIL.

	Replicates of sample				Replicates of crude protein			
	Non-treated soil		Treated soil		Non-treated soil		Treated soil	
	1	2	1	2	1	2	1	2
	%	%	%	%	%	%	%	%
Nitrogen	3.09	2.92	3.39	3.30				
Protein (N×6.25)	19.31	18.25	21.19	20.63				
Methionine	.17	.16	.20	.19	.88	.88	.94	.92
Lysine	.67	.61	.72	.66	3.50	3.34	3.40	3.20
Valine	.94	.93	1.03	1.01	4.87	5.10	4.86	4.90
Leucine	.89	.81	.96	.90	4.61	4.44	4.53	4.36
Arginine	.85	.76	1.06	.94	4.40	4.16	5.00	4.56
Histidine	.23	.21	.32	.32	1.19	1.15	1.51	1.55
Phenylalanine	.50	.47	.57	.56	2.59	2.58	2.69	2.71
Isoleucine	.30	.34	.36	.33	1.55	1.86	1.70	1.60
Threonine	.73	.66	.75	.69	3.78	3.62	3.54	3.34
Tryptophan	.17	.16	.20	.19	.88	.88	.94	.92

basis of percentage of protein in the sample indicate that there is an accumulation of protein. The differences in amino acids are not as pronounced as was noted in the tissue treated with 2,4-dichlorophenoxyacetic acid (**8, 12**). The results expressed as per cent. of amino acid in the crude protein suggest that the nature of the protein has changed since the percentage of amino acids are, as a rule, less in the soil-treated tissue than in the controls. This would indicate that the TCA has an effect on the metabolism of proteins and amino acids.

The results in table II show only a slight increase in the percentage of protein and amino acids in the root tissue of the soil-treated plants. When expressed as percentage of the crude protein, little difference was observed in most of the amino acids with the exception of histidine and arginine which was slightly greater in the plants grown in the treated soil. The data

TABLE III

CARBOHYDRATE AND ETHER EXTRACT CONTENT OF SHOOTS FROM WHEAT PLANTS GROWN IN SODIUM TRICHLOROACETATE TREATED AND NON-TREATED SOIL.

	Non-treated replicates		Treated replicates	
	1	2	1	2
	%	%	%	%
Reducing sugar	2.90	2.93	3.20	3.58
Non-reducing sugar	8.20	8.49	2.74	2.92
Starch	0.65	0.72	1.04	1.26
Other polysaccharides*	9.20	8.62	10.83	10.72
Ether extract	5.37	5.37	2.33	2.47
Unsaponifiables**	1.02	1.05	0.51	0.54
Fatty acids**	1.57	1.27	0.84	0.83

*Acid hydrolyzable residue from starch determinations.
**Chlorophyll free.

in table III show a slight increase in the percentage of reducing sugar and acid-hydrolyzable polysaccharides in the shoots of the soil-treated wheat plants. On the other hand, a large decrease was noted in the percentage of non-reducing sugar, ether extract, unsaponifiable material, and fatty acids. An increase was observed in the percentage of starch. In the roots (table IV) there was a higher percentage of reducing sugar and starch in the soil-treated plants than in the controls. Little difference was noted in the percentage of the non-reducing sugar and ether extract as a result of soil treatment. Only a slight reduction was observed in the acid-hydrolyzable polysaccharide due to soil treatment. The decrease in the percentage of non-reducing sugar in the shoots, the large increase of reducing sugar and starch in the roots, and the accumulation of protein in the shoots of the TCA-soil-treated plants suggest that the depletion of non-reducing sugar in the shoots results from its partial utilization in protein synthesis and the

TABLE IV

CARBOHYDRATE AND ETHER EXTRACT CONTENT OF ROOTS FROM WHEAT PLANTS GROWN IN SODIUM TRICHLOROACETATE TREATED AND NON-TREATED SOIL.

	Non-treated replicates		Treated replicates	
	1	2	1	2
	%	%	%	%
Reducing sugar	3.82	3.83	6.63	7.86
Non-reducing sugar	3.09	3.44	3.37	3.45
Starch	2.51	1.67	4.59	5.41
Other polysaccharides*	20.95	21.05	16.89	16.24
Ether extract	2.86	3.02	2.18	2.33

*Acid hydrolyzable residue from starch determinations.

TABLE V

RESPIRATORY ACTIVITY OF SHOOT AND ROOT TISSUE FROM WHEAT PLANTS GROWN IN SODIUM TRICHLOROACETATE TREATED AND NON-TREATED SOIL AS MEASURED IN A WARBURG RESPIROMETER.

Day	μl. Oxygen/hr./mg. fresh weight*				Ratio of treated to control	
	Control tissue		Treated tissue			
	Leaves	Roots	Leaves	Roots	Leaves	Roots
10	0.39	0.17	0.50	0.36	1.28	2.12
20	0.47	0.18	0.53	0.28	1.13	1.56

*Average of three determinations.

remainder possibly being translocated to the root tissue in the form of reducing sugar and stored as starch.

Further evidence of accelerated metabolism in the TCA-soil-treated shoots and roots was apparent from the data obtained in respiratory measurements (table V). The oxygen uptake was greater in the soil-treated plants at the end of a 10- and 20-day period. No increase in oxygen uptake was observed in plants grown three days in the TCA-treated soil. The ratio of the treated to the control was approximately the same in the shoots at the end of 10 and 20 days. In the root tissue a decrease was noted in the ratio at the end of a 20-day period.

Summary

The shoots of wheat plants grown in soil treated with the sodium salt of trichloroacetic acid contained more protein, arginine, reducing sugar, and acid-hydrolyzable polysaccharide on a per cent. basis. Slight differences were noted in the other amino acids and starch with the exception of tryptophan which was less in the soil-treated samples. A decrease was observed in the percentage of non-reducing sugar, ether extract, unsaponifiable material, and fatty acids of the soil-treated plants. The data expressed as per cent. of amino acid in the crude protein showed approximately the same amount of methionine and arginine and slightly less of the other amino acids.

The roots of plants grown in soil treated with sodium trichloroacetate showed a slight increase in the percentage of proteins and amino acids and also an increase in reducing sugar and starch. Little difference was noted in the amino acids (expressed as percentage of crude protein) with the exception of histidine and arginine which were slightly greater in the soil-treated plants. The presence of sodium trichloroacetate in the soil did not greatly alter the percentage of non-reducing sugar and ether extract.

Respiratory activity was greater in both the shoots and roots of the plants grown in soil treated with sodium trichloroacetate.

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AUXIN RELATIONS OF A DWARF PEA

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Introduction

Inheritable dwarfness in higher plants is generally believed to be related to measurable auxin metabolism. VAN OVERBEEK (**12, 13**), who investigated six of the dwarfs of maize, found in each case a lower production of auxin in the dwarf than in the normal sib. The destruction of auxin in the nana dwarf was compared with that in the normal sib, and was found to be greater than in the normal. This higher auxin destruction was concluded to be the cause of the reduced growth in the nana dwarf. HINDERER (**7**) was able to correlate the degree of dwarfness of Epilobium hybrids inversely with auxin production. DE HAAN and GORTER (**5**) compared a tall-stemmed variety with a short-stemmed variety of Pisum and found the short-stemmed variety able to inactivate a greater quantity of auxin than the tall.

There are, however, various characteristics associated with dwarfism in maize (**6**) which make it difficult to accept these simple differences in auxin metabolism as its fundamental cause. At least 20 distinct dwarfs of maize have been recorded (**6**). Those for which genetic data are available are simple recessives, and the loci involved have been located on seven of the ten chromosomes. Several of these dwarfs (an_1, an_2, d_1, d_4) produce stamens in the pistillate inflorescence, while another (er_1) bears seed on the staminate tassel. An abnormal number of tillers is commonly produced, and leaves may vary from the normal in texture, thickness, area, and shape. The remarkable range of variation in type of dwarfing is apparent in the following group: internodes uniformly shortened (an_1), internodes uniformly shortened but with other organs of normal size (br), all internodes shortened except the one immediately below the tassel (na_1), internodes immediately below the tassel most shortened (d_6), and internodes immediately above and below the ear shortened, with others of normal length (bv). DE HAAN and GORTER (**5**) have observed that the short and long-stemmed peas used in their investigation also differed in many characteristics other than longitudinal growth.

It would seem worthwhile to extend auxin investigations to other varieties within these two species, and to other species as well. If the concept is generally verified, it would be desirable to elucidate the mechanics of the causal reaction, and to investigate the pathway by which the divergent expressions of the reaction are reached.

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Materials and methods

These studies were made with two commercial varieties of Pisum, Telephone and Dwarf Telephone. Etiolated seedlings were used throughout the investigation. They were found to be more readily adapted to experimental techniques than green seedlings, and the difference in height between dwarf and tall is more pronounced and more quickly attained in the etiolated state. Seeds were sterilized in 0.5% NaOCl for five minutes and germinated in sand in a dark room (25° C) at high humidity. Low intensity red light was used for all necessary illumination.

Two methods of auxin determination were employed. A colorimetric test, with the Salkowski $FeCl_3$ reagent as modified by TANG and BONNER (**10**), was used to determine residual indoleacetic acid (IAA). An aliquot of the experimental solution (2 ml.) was added to 8 ml. of the color reagent, and the color intensity was read in a Klett colorimeter after a 30-minute development period at room temperature.

The standard Avena test (**14**) was used to measure the decrease in biological activity of extrinsic IAA, and also to estimate quantities of native plant hormone. Experimental solutions were extracted with purified ether, the ether evaporated off on a water bath, and the test material made up to 0.8 ml. with 1.5% to 2.5% agar. Twelve test blocks were prepared from this solution, each 11.9 cu. mm. in volume. Three standardized controls were used and will be indicated by letter in this report as follows: type A, 0.08 γ IAA made up to same volume as the experimental sample with the appropriate buffer, incubated for three hours, extracted as usual and the dried extract made up to 0.8 ml. with 2% agar; type B, 0.08 γ IAA made up to 0.8 ml. with 2% agar; type C, 0.04 γ IAA made up to 0.8 ml. with 2% agar. The concentration of the type B control (100 γ per liter) is usually just below the upper limit of the proportionality range of dilution for the Avena test under the conditions of this laboratory. Control A is used to reveal any loss of activity caused by the method of handling test solutions, and controls B and C are used to establish the sensitivity of the test plants. Each value reported for the Avena test is the average of 12 test plants.

Results

A comparison of the growth habit of the dwarf and tall etiolated pea seedlings is shown graphically in figure 1. Height of the stems was measured from soil level to the lowest leaf-scale of the apical bud. Weight relationships of the dwarf and tall etiolated seedlings, 12 days after planting, are shown in table I. It is evident that the dwarf plants grow approximately one half as rapidly in height as do the tall plants. Differences in weight are less pronounced. The problem is to determine whether or not this quantitative difference in growth is related to a difference in auxin metabolism. If the growth of the dwarf is limited ultimately by a low level of available auxin, it should be possible to overcome the limitation by providing the plant with additional growth hormone.

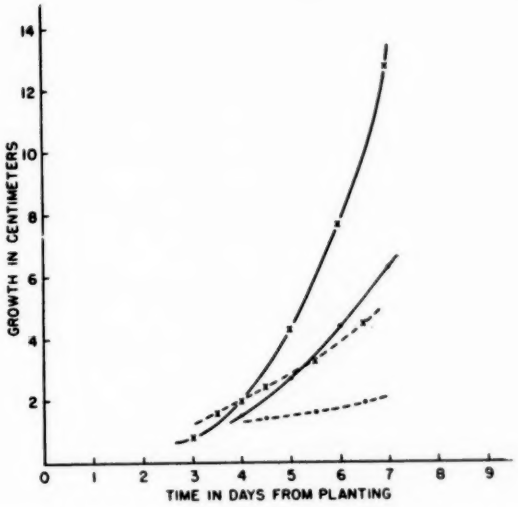

FIG. 1. Growth of dwarf and tall etiolated pea seedlings. Solid line: growth as height of stem of intact etiolated plants. Broken line: rate of growth as increment per 24 hours. Averages of 36 plants. Tall: ——×——; dwarf ——•——.

EFFECT OF IAA ON GROWTH OF INTACT ETIOLATED SEEDLINGS

Dwarf and tall seedlings were grown in groups of 12. Six days from the time of planting, the etiolated seedlings were sprayed with IAA at a concentration of 2 mg. per liter. Duplicate groups of control plants were sprayed with distilled water. Both IAA and control spray solutions contained a small quantity of wetting agent. The treatment was repeated 22 hours after the initial spraying. The entire experiment was conducted in a dark room maintained at 24 to 27° C and 70 to 80% humidity. Low intensity red light was used when necessary. Response was measured as the increase in height of the seedling from the time of the first application. Measurement was made from soil level to the lowest leaf-scale of the apical bud. The results

TABLE I

WEIGHTS OF DWARF AND TALL ETIOLATED PEA SEEDLINGS 12 DAYS AFTER PLANTING. AVERAGES OF 12 PLANTS.

	Height	Roots			Tops		
		Fresh weight	Dry weight	Dry weight	Fresh weight	Dry weight	Dry weight
	cm.	*mg.*	*mg.*	%	*mg.*	*mg.*	%
Tall	27.6	282	25	8.9	1072	62	5.8
Dwarf	15.0	260	21	8.1	811	54	6.7
Dwarf / Tall	54.3%	92.2%	84.0%		75.5%	87.1%	

shown graphically in figure 2 represent the average of three groups of 12 plants each. It may be seen that the auxin treatment increased the growth rate of the dwarf, but inhibited the growth rate of the normal plant. Increasing the concentration of IAA to 3 mg. per liter and increasing the frequency of application did not increase the measured response.

If this difference in response to exogenous IAA is to be analyzed, it is necessary to define its cause in terms of the over-all auxin metabolism of the two varieties. In order to do so, data must be assembled concerning as many phases of the auxin metabolism as are amenable to direct attack. In

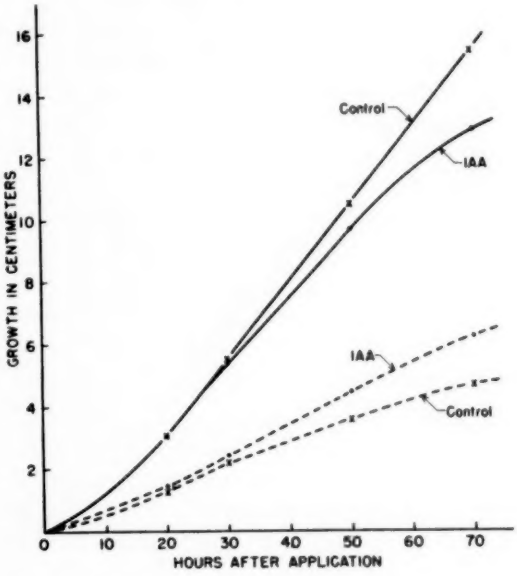

FIG. 2. Influence of IAA on growth of etiolated dwarf and tall pea seedlings. Applied as a spray, 2.0 mg. per liter. Increase in height from time of application. Average of 36 plants. Solid line: tall. Broken line: dwarf.

this effort, experiments have been designed to measure auxin inactivation, diffusion of auxin, content of free and combined auxin and ability to produce auxin from tryptophane.

INACTIVATION OF IAA BY MACERATED EPICOTYL

COLORIMETRIC METHOD.—Epicotyls of specified age were harvested and washed. They were macerated in a chilled blender in the cold room with the use of cold citrate-phosphate buffer, 0.1 to 0.2 molar, pH 6.8 or 7.0. This preparation was filtered through coarse filter paper under suction. The filtrate was made to volume with buffer, and aliquots withdrawn for the reaction mixtures. Each reaction mixture was made up of 20 ml. of the filtrate

plus 20 ml. of IAA solution, 20 mg. per liter. A control of filtrate plus buffer was prepared, primarily to provide a correction for the increase of turbidity during the incubation periods. Aliquots of 2 ml. were withdrawn from the reaction mixture at specified periods and mixed with the color reagent. The colorimeter reading on the turbidity blank was subtracted from the experimental reading, and the residual IAA in the aliquot estimated from a standard curve. The number of epicotyls and the fresh weight of tissue represented by each reaction mixture was calculated as a fraction of the original filtrate. In table II, data have been included to show the effect of seedling age. Auxin destruction as measured in this way appears to be nearly identical in dwarf and tall plants. Similarly, separation of epicotyls into apical and basal halves failed to reveal any difference between dwarf and tall. No inactivation was found after heat denaturation of the filtrate.

TABLE II

INACTIVATION OF IAA BY FILTRATE OF MACERATED EPICOTYLS OF DWARF AND TALL PEA. COLORIMETRIC DETERMINATION. INITIAL CONCENTRATION OF IAA. 10 MG. PER LITER.

	Age	IAA inactivated			
		Per gram tissue	Per epicotyl	Per gram tissue	Per epicotyl
	days	*μg./1 hr.*		*μg./3 hrs.*	
Tall	5	47	15	60	20
Dwarf	5	47	15	61	20
Tall	7	35	17	56	28
Dwarf	7	34	16	53	25
Tall	10	30	26	40	35
Dwarf	10	32	25	43	34

AVENA TEST.—Etiolated epicotyls were macerated at low temperature and filtered in the same manner as for the colorimetric method. IAA was added to the filtrate and the mixture made up to volume. In some experiments an excess of IAA and a long incubation period were employed, after which the reaction mixture was heated to boiling, acidified to pH 3.0 (glass electrode) and extracted with ether. Serial dilutions were then made from the extract. It was found more satisfactory, however, to add to the reaction mixture a quantity of IAA near the upper limits of the proportionality range of the Avena test, and then to hold three aliquots of standard volume for incubation periods of zero, one, and three hours, respectively. This procedure was followed, after denaturation, by acid-ether extraction without subsequent dilution of the extract. With this method, the zero-time aliquots are expected to give a curvature approximately equal to that of control type B. The relative IAA inactivating potentials of seven-day-old dwarf and tall etiolated peas, as determined by the method outlined, are shown in table III. Again auxin destruction is nearly identical in the two forms. Plants five days old or 10 days old showed the same similarity between the

dwarf and tall. Heat denaturation of the crude filtrate was found to eliminate effectively the inactivation of IAA. This result is in agreement with that of the colorimetric method.

In order to compare the pH optima of the inactivation in dwarf and tall plants, fresh tissue was ground in glass-redistilled water and the filtrate was

TABLE III

INACTIVATION OF IAA BY ETIOLATED DWARF AND TALL PEAS SEVEN DAYS OF AGE. FILTRATE OF MACERATED EPICOTYLS, DETERMINATION BY AVENA TEST, INITIAL CONCENTRATION OF ADDED IAA: 4.0 MG. PER LITER.

	Incubation period	IAA in degrees of curvature	Inactivation in degrees of curvature	Inactivation in curvature per gram fresh weight
	hrs.			
Control A	3	16.9 ± 0.36		
Control B	..	18.5 ± 0.47		
Control C	..	9.8 ± 0.39		
Tall	0	16.9 ± 0.43	....	....
	1	5.9 ± 0.38	11.0	14.9
	3	0	....	....
Dwarf	0	17.1 ± 0.59	....	....
	1	6.3 ± 0.39	10.8	15.2
	3	0	....	....

lyophilized. Portions of the lyophilate were taken up in phosphate-citrate buffer at each pH level. The IAA was added, the reaction mixture made up to volume with phosphate-citrate buffer, and the pH adjusted. The mixture was then incubated for one hour at room temperature and the remaining IAA determined by the Avena test. The IAA destruction by both the dwarf and tall plants was greatest over the pH range 6.0 to 6.8.

Inactivation of IAA by epicotyl sections

Terminal buds were removed from etiolated seedlings seven days after planting. One hour after the decapitation, a 2-mm. section of epicotyl was excised from each seedling at a point 2 mm. below the previous cut. Each section was immediately placed, base down, upon an agar block containing 100 γ IAA per liter. Contact between the agar block and the basal cut surface of the epicotyl section was maintained for one half, one, and three hours, respectively, for three separate groups of 12 test blocks each. The entire experiment was conducted in darkness interrupted when necessary only by low intensity red light. Over the three-hour period, close control of humidity and temperature was necessary to avoid drying of the agar. The IAA remaining in the test blocks was determined by the Avena test. The data in table IV compare IAA destruction by dwarf and tall plants, as shown by this method, and indicate no difference in the two forms.

TABLE IV

INACTIVATION OF IAA BY EPICOTYL SECTIONS. DETERMINATION BY AVENA TEST. INITIAL CONCENTRATION IAA: 100 γ PER LITER.

	Contact period	Remaining IAA in degrees of curvature	Inactivation of IAA in degrees of curvature
	hrs.		
Control B	....	17.7 ± 0.56	
Control C	....	8.9 ± 0.69	
Tall	.5	17.5 ± 0.72	0.2
	1	15.1 ± 0.80	2.6
	3	11.6 ± 0.79	6.1
Dwarf	.5	18.0 ± 0.64	0
	1	14.7 ± 0.84	3.0
	3	12.1 ± 0.56	5.6

AUXIN DIFFUSION FROM EXCISED APICAL BUDS

Apical buds were removed from etiolated dwarf and tall pea seedlings by cutting with a fine blade at a point 2 mm. below the lowest leaf-scale of the bud. The excised buds were immediately mounted between two glass plates, as described by VAN OVERBEEK (11). Six buds were mounted between each pair of plates, and the whole assembly placed in a Petri dish lined with wet filter paper. A standard size Avena test block (3% agar) was applied to the cut surface of each bud. After a specified period of time, the agar blocks were removed and the auxin content of each block was determined by the Avena test. The test blocks desiccate rapidly even at 90% humidity, and the accuracy of the method is easily destroyed. Therefore the test blocks were removed after each one-hour period and fresh blocks were immediately installed. Low intensity red light was used for necessary manipulation. Table V shows that the quantities of auxin obtained from the two types of plants seven days old were remarkably similar. This similarity between dwarf and tall was found to exist as well in younger and older plants, five and 10 days of age. Apical buds of the plants 10 days old, however, diffused less auxin than the younger ones.

TABLE V

AUXIN DIFFUSED FROM ABSCISED APICAL BUDS OF ETIOLATED PEA SEEDLINGS SEVEN DAYS OF AGE, AVENA TEST, AVERAGES OF 12 BUDS.

	Auxin in degrees of curvature			
	First hour period	Second hour period	Third hour period	Fourth hour period
Control B	18.3 ± 0.66			
Control C	10.8 ± 0.61			
Tall	12.7 ± 0.71	10.3 ± 0.53	3.8 ± 0.37	1.3
Dwarf	13.2 ± 0.59	19.7 ± 0.50	4.3 ± 0.39	1.5

Ether extractable auxin

Etiolated epicotyls were harvested seven days after planting, washed in distilled water and rinsed in ether. They were then held under an excess (approximately 400 ml.) of purified ether for 20 hours at 0 to 2° C. The ether was decanted off, and the epicotyls washed with two small portions of fresh ether. The combined solvent was distilled off, and the residue made up to 0.8 ml. with 2% agar. Serial dilutions were made from this preparation, and the extracted auxin determined by the Avena test. Auxin contents of the dwarf and tall, as measured in this way, were essentially identical. The values shown in table VI are substantially values of the free auxin content of the tissue since the low temperature used for extraction is known to minimize auxin production during the extraction period.

TABLE VI

AUXIN EXTRACTABLE FROM INTACT ETIOLATED EPICOTYLS OF DWARF AND TALL PEAS WITH COLD ETHER, PERIOD: 20 HOURS. DETERMINATION BY AVENA TEST.

	Number of epicotyls extracted	Dilution	Auxin in degrees of curvature	Auxin in degrees of curvature per gram fresh weight
Control A			16.3 ± 0.41	
Control B			17.1 ± 0.43	
Control C			8.8 ± 0.36	
Tall	20	1	17.3 ± 2.23	....
		.5	15.8 ± 0.79	2.8
		.25	8.0 ± 0.63	2.8
Dwarf	20	1	16.2 ± 1.73	....
		.5	16.2 ± 0.51	3.0
		.25	8.6 ± 0.55	3.2

Auxin released by alkaline hydrolysis

Etiolated epicotyls were harvested seven days after planting, macerated in a blender with phosphate-citrate buffer at pH 7.0, and filtered. The filtrate was placed under a layer of toluene, allowed to remain at room temperature for three hours and was then lyophilized. Samples of the lyophilate were taken up in 0.05 molar BO_3-KCl buffer at pH 9.6 and then either held in a boiling water bath for 30 minutes or refluxed for 15 hours. The hydrolysate was cooled, acidified to pH 3.0 and extracted with ether. The ether was distilled off and the auxin content of the residue determined by the Avena test (table VII). Auxin levels before hydrolysis were not determined for this experiment, but approximate equality of dwarf and tall may be assumed from previous determination. No difference in auxin content was revealed.

Conversion of tryptophane to auxin

Two random samples each of dwarf and tall etiolated epicotyls, seven days of age, were cut into two-inch lengths. One sample of each variety was

TABLE VII

AUXIN RELEASED FROM ETIOLATED EPICOTYLS OF DWARF AND TALL PEAS UNDER ALKALINE HYDROLYSIS. DETERMINATION BY AVENA TEST.

	Lyophilized tissue	Method of hydrolysis	Dilution	Auxin in degrees of curvature	Auxin in degrees of curvature per gram fresh weight
	mg.				
Control B				16.2 ± 0.65	
Control C				8.4 ± 0.66	
Tall	200	Half hour bath	.5	18.7 ± 0.73	5.9
			.25	9.4 ± 0.77	5.9
Dwarf	200	Half hour bath	.5	19.3 ± 0.73	6.3
			.25	8.9 ± 0.65	5.9
Control B				17.5 ± 0.65	
Control C				8.2 ± 0.62	
Tall	200	15 hours reflux	.33	20.0 ± 1.00	9.5
			.1	7.7 ± 0.60	12.2
Dwarf	200	15 hours reflux	.33	21.1 ± 1.19	10.5
			.1	7.1 ± 0.59	11.8

immersed in 20 ml. of 0.1 M phosphate buffer, pH 7, and the second sample was immersed in 20 ml. of the same buffer containing 10 mg. of tryptophane. The samples were infiltrated under low pressure for 30 minutes and then incubated under toluene at 22° C for three hours. Each sample was rinsed with distilled water and held under 250 ml. of purified ether at 0 to 2° C for 20 hours. The ether was decanted off, and the tissue rinsed with small volumes of fresh ether. The supernatant and washings from each sample were combined, and the ether distilled off. The auxin content of the residue was

TABLE VIII

CONVERSION OF TRYPTOPHANE TO AUXIN BY ETIOLATED EPICOTYLS OF DWARF AND TALL PEAS. DETERMINATION BY AVENA TEST.

	Concentration of tryptophane in medium	Dilution	Auxin in degrees of curvature	Estimated conversion in degrees curvature	Estimated conversion in curvature per gram fresh weight
	mg./ml.				
Control B			16.2 ± 0.82		
Tall	0	1	7.8 ± 0.64		
		.5	3.5 ± 0.49		
	0.5	1	18.8 ± 0.60	11.0	4.0
		.5	9.4 ± 0.57	11.0	4.0
Dwarf	0	1	7.0 ± 0.45		
		.5	2.8 ± 0.45		
	0.5	1	17.9 ± 0.86	10.9	4.3
		.5	9.3 ± 0.84	11.6	4.6

determined by the Avena test. The results (table VIII) do not indicate a significant difference in the ability of the two varieties to convert tryptophane to auxin.

Discussion

The degree of dwarfness of the pea variety Dwarf Telephone is established by the growth data given in figure 1 and table I. The height of the dwarf is roughly only half that of the tall. In contrast, the fresh weight of the dwarf epicotyl is 75% that of the tall, and the dry weight nearly 90%. This is precisely the sort of relationship which might be predicted for an auxin deficiency. There are numerous conceivable aberrations in auxin metabolism which might reasonably be expected to result in dwarfism. The most obvious of these are an exaggerated destruction of the auxin molecule without contribution to the growth process, a subnormal capacity for auxin synthesis, an inability to utilize auxin in normal quantities, and an abnormal competitive requirement for auxin. If the first of these hypothetical situations in fact exists, the inactivation of the hormone should easily be proved to be greater in the dwarfed plant than in its tall sib. Tang and Bonner (**10**) have demonstrated the enzymatic inactivation of IAA in one variety of pea seedlings, and Larsen (**8**) has shown that an enzyme capable of inactivating IAA occurs in bean seedlings. Bonner and Wildman (**3**) mention the occurrence in plant tissues of an inhibitor of this enzyme system. Thus, an excessive destruction of IAA in the dwarf might be a result either of an effective increase in an enzyme system, or a low inhibitor level for that system.

Van Overbeek (**12**) concluded that the restricted growth of the nana dwarf of maize is the consequence of a large destruction of growth substance, and that the high rate of destruction, as compared to the normal sib, may reflect the oxidation-reduction situation in the two varieties. Similarly, De Haan and Gorter (**5**), from their investigation of a short-stemmed pea, concluded that auxin is inactivated in this organism by oxidizing enzymes and that the length of stem of the variety is determined by its content of the enzymes.

Results of the present investigation, however, fail to show that the dwarf and tall pea seedlings differ significantly in their ability to inactivate IAA. Studies using both the colorimetric determination of IAA and the Avena test indicate that the system may be inactivated by heat, and that it shows an order of activity and a pH optimum similar to that reported by Tang and Bonner (**10**). Both the dwarf and tall pea have a pH optimum for the inactivation in the range of 6.0 to 6.8. It is of interest to note that the measurable inactivation of IAA decreases as the age of the plant increases from five to 10 days and that the basal half of the epicotyl is a less productive source of the enzyme than is the apical half. Although studies of macerated tissues do not conclusively define the normal regulatory system of the intact plant, in this case it may be assumed that no substantial differ-

ence of the type under consideration is masked by the disorganization. This assumption is substantiated by use of the technique utilized by VAN OVERBEEK (**12, 13**), and by DE HAAN and GORTER (**5**), in which an excised section of epicotyl is placed in contact with known quantities of IAA. The resultant inactivation does not indicate any distinction between the dwarf and the tall. It is important that the epicotyl sections for this procedure are taken only from the growing zone, since any inactivation of auxin which is to directly affect growth in length of these seedlings must occur in or above this region.

The possibility of an aberrant auxin synthesis in the dwarf plant has been considered by previous workers. The production of auxin by coleoptile tips of dwarf maize was determined by VAN OVERBEEK (**12, 13**) with the diffusion method. With plants four days of age, more growth substance was obtained from coleoptile tips of the tall seedlings than from those of the dwarf. By the age of seven days, however, this difference was no longer discernible in three of the dwarfs tested, nana, dwarf 2, and pigmy. DE HAAN and GORTER (**5**) used the same method to determine the auxin production of the terminal buds of a long and a short-stemmed pea. Measurements for the first two and one half hours of diffusion indicate that the dwarf actually produced more auxin than the tall sib during that period. From the evidence presented, it appears very doubtful that the total quantity of auxin obtained from the tall pea during the four and one half hours of the experiment would equal that from the dwarf. It would also seem that the normal situation within the plant would be more accurately represented by the earliest periods of this type of experiment. The assumption that a high rate of auxin destruction in the dwarf overcomes its initial superiority in production is highly questionable.

In the present investigation, the diffusion method has failed to demonstrate any difference between dwarf and tall peas in the production of auxin at an age of five, seven, or 10 days. Measurable diffusion of auxin was found to decrease rather sharply over four successive one-hour periods following isolation of the apical buds. WENT and THIMANN (**14**) have stated that only small amounts of auxin are obtainable from the apical buds of Pisum by diffusion, and in this regard the present results are a noteworthy exception. In considering results of the diffusion method, it is prudent to observe that both auxin synthesis and auxin release may be involved.

The content of free growth substance at a given time must be the consequence of a number of processes: the sum of synthesis and release less the quantities utilized, destroyed and bound. An effective divergence of the subject organisms in the course of any one of these contributing processes may be expected to be disclosed by determination of the free auxin content. For this purpose, a 20-hour, cold ether extraction of intact epicotyls was used. The dwarf and tall evidently contain nearly identical quantities of free auxin. Calculation from data given in table VI indicates a total extraction of auxin roughly equivalent to 150° of Avena curvature per epicotyl, as

compared to a measurable diffusion of approximately 10° per apical bud per hour for both dwarf and tall. Certain enzymatic activity is expected to continue at a low rate under the conditions of these extractions, and it is probable that synthesis, release and inactivation of auxin all occur to a slight extent during the extraction period. Regardless of these activities, the data so obtained may be assumed to approximate the relative auxin contents of the two organisms at this availability level. This equality is in agreement with the data on production and inactivation of the hormone, but is not easily reconciled with the differential response to exogenous IAA.

Wildman *et al.* (**16**) have demonstrated an enzyme system in spinach leaves capable of converting tryptophane to an active growth substance, probably IAA, and it is suggested by these authors that this may well be the final step in the biosynthesis of a native auxin. It is interesting to observe that a similar conversion does occur in both the dwarf and the tall pea. There is no evidence, however, that the two varieties differ in their respective capacities for this conversion.

Auxin is known to occur in various plant tissues in an incorporated or bound state, in contrast to the free auxin which is active in the Avena test. Avery *et al.* (**1**) and Berger and Avery (**2**) have shown that relatively large quantities of IAA can be released from corn seed by alkaline hydrolysis. Skoog and Thimann (**9**) found an increase in auxin in Lemna after enzymatic proteolysis. Wildman and Gordon (**17**) demonstrated that the auxin of spinach leaves is associated with proteins, and may be released by tryptic hydrolysis. Wildman and Bonner (**15**) have further elucidated the nature of the auxin-protein relationship. Epicotyls of the dwarf and tall pea yield substantial quantities of growth substance upon alkaline hydrolysis, but it is apparent that the two do not differ in this respect. All pertinent evidence thus seems to justify the assumption of a strict quantitative similarity between the dwarf and tall in their individual capacities for auxin synthesis.

The manner in which auxin is utilized by the plant to produce growth is not known, and it is necessary to consider the possibility that an incorporated form of the hormone may be the functional one. Bonner and Wildman (**3**) have proposed that this may be the case, specifically for the protein-bound auxin. Any aberrant situation inhibiting the formation of the auxin-protein might thus interfere with the growth process, and a depressed growth rate could occur even in the presence of an adequate supply of free auxin. The present investigation has shown that alkaline-hydrolyzable auxin occurs in approximately equal quantities in the dwarf and tall pea. It has not been shown, however, that this released auxin represents, in each case, the same type of combination. The possibility remains that a large fraction of the bound auxin in the dwarf may occur as some inactive complex, with only a small remainder representing the active auxin-protein.

Regardless of the form in which auxin exerts its influence, or of the mechanism of the action itself, a competition for the free auxin molecule at normal availability levels would be expected to result in a diminution of growth. It is, therefore, necessary to consider the hypothetical circumstance

in which the growth reaction in the dwarf must compete with some undefined system for available auxin. The results of the present investigation provide limited evidence both for and against the possibility of such a situation. It is apparent that the auxin productivities of the dwarf and tall pea are very similar, if not identical, and also that the capacities for IAA inactivation are equal. Presumably, then, a strong competitive demand for auxin in the dwarf would reduce the level of the free hormone to a point significantly below that in the tall pea. Both ether extraction and diffusion of auxin have failed to reveal a difference of this nature. The investigation, however, has demonstrated a difference in response of the two organisms to IAA applied as a spray to the intact growing plants. At a concentration of 2 mg. per liter, the growth of the dwarf shows an increase of about 30% over that of the control, while the growth of the tall pea is significantly repressed. The facts suggest that an active demand for IAA by the dwarf has been complied with, while toxic levels have been attained in the tall. It is difficult to visualize a situation in which, in the dwarf pea, the supply of free Avena-active growth substance is not depleted in the presence of an unsatisfied requirement for IAA. At the present time, there is no justification for postulating functional differences in the various auxins. Although an IAA response differential has been established, possibly reflecting a highly competitive situation in the dwarf pea, it has not been possible to induce the growth rate of the dwarf to approach that of the tall, even with higher concentrations and more frequent application of auxin. The seemingly higher IAA requirement of the dwarf is evidently a secondary difference, and not the fundamental cause of dwarfism. An interesting contrast is found in the results of VAN OVERBEEK (**12**), where the curvature response of tall maize to auxin applied unilaterally was greater than that of the nana dwarf.

Summary

The investigation has shown that intact dwarf and tall etiolated pea seedlings, variety Telephone, exhibit a differential response to applied IAA. Spray application of a 2 mg. per liter IAA solution to plants six days of age resulted in a 30% increase in growth of the dwarf, as compared to the control, while growth of the tall variety was significantly repressed. Higher concentrations and more frequent applications did not increase the differential response. Efforts to relate this differential response to some particular aspect of the auxin metabolism were not successful. The two varieties are shown to give remarkably similar results to the following measurements: inactivation of IAA by macerated epicotyls as determined both colorimetrically and by the Avena test, approximate pH optima of the inactivation of IAA, inactivation of IAA by sections of epicotyl, diffusion of auxin from excised apical buds, content of free auxin by cold ether extraction, release of auxin from epicotyls under alkaline hydrolysis, and the conversion of tryptophane to auxin by epicotyl sections. It is suggested that the difference in response to exogenous IAA may not in itself indicate the fundamental cause of dwarfism in this variety.

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THE ROLE OF BORON IN THE TRANSLOCATION OF SUCROSE[1]

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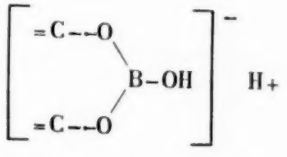
Introduction

The essentiality of boron for higher plants was demonstrated (**1**) in 1910 and extensively verified in the two succeeding decades. Since then a voluminous literature has accrued concerning the anatomical, morphological, and biochemical alterations of boron-deficient plants. Inasmuch as they are not pertinent to the following report, many of the specific or general roles that have been postulated for boron will not be discussed. By their omission the authors do not mean to imply that boron has no role or roles other than the one presented since, for example, the inverse relationship between boron level and per cent. moisture in plant tissues has been firmly established by MINARIK and SHIVE (**15**) and others. To date, however, no one has demonstrated the exact role or mode of action of boron in higher plants. It is the purpose of this report to offer evidence for one role of boron.

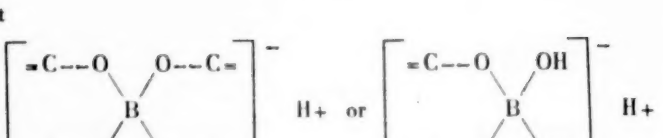
In view of the well-known reaction of the borate ion with OH-rich compounds and its recent application in the borating of sugars in order that they may be separated on ion-exchange columns (**14**, **18**), the authors conceived and tested an hypothesis as to one essential role of boron in plants. The hypothesis is that boron combines with sugar to form a sugar-borate complex (ionizable) which is translocated with greater facility than are non-borated, non-ionized sugar molecules. Inasmuch as most studies have indicated that sucrose does not move readily through cellular membranes, the work reported herein was limited to sucrose and the effect of a few (5 to 10) parts per million of boron on its translocation.

According to ZITTLE (**26**) the borate ion reacts most strongly with OH-rich compounds in which there are two OH groups in the cis position. Although the structure of sucrose does not fulfill this requirement, there is ample evidence that there is a reaction with sucrose and that it involves the trans-1,2-diol groups. Further (**26**), it is suggested that the following structure is formed with sucrose



[1] Contribution no. 2386 of the Maryland Agricultural Experiment Station, Department of Botany.

and not



as would be formed with certain sugars and other carbohydrates, the form or forms varying with different conditions such as changes in concentration and pH.

Methods and results

The first experiments to test the hypothesis involved the Warburg apparatus with which the effect of boron on the rate of entry and translocation of the substrate (sucrose) could be studied. With this apparatus it was possible to measure the O_2 uptake of excised root tips. Some of these received, in addition to the base nutrient salts, only sucrose and others received sucrose plus 5 p.p.m. of boron as boric acid. Boron alone, at this concentration, had no effect on the respiration of the root tips used in these studies.

In certain cases the root tips did not respond to added sucrose owing to an internal source of carbohydrate (starch). Therefore, in order to get a response to added sucrose it was sometimes necessary to excise the root tips from the germinating pea or lima bean seeds and to culture the tips in an oxygenated base nutrient solution for 18 hours to effect a comparatively low level of endogenous respiration. In all cases in which the root tips did respond to an exogenous source of sucrose, without exception a greater respiration (O_2 uptake) occurred when 5 p.p.m. of boron was present in the sucrose and base nutrient solution. Increases in respiration of 50 to 85% were observed after 10 hours (fig. 1 A, B, C and D) with greater percentage increases in the earlier part of certain experiments.

It should be borne in mind that these responses were obtained with root tips that were presumably adequately supplied with boron from the cotyledons. In various experiments the increases in respiration in the presence of boron did not occur with the same concentration of sucrose. It is suggested that the metabolic status of the root tips at the time they were used caused this variation with respect to the particular level of sucrose in which the boron effect occurred (fig. 1 A) or was most striking (fig. 1 D). It would appear from the results that factors other than sucrose were limiting in certain of the experiments. When this situation exists, the boron effect with the higher level of sugar cannot possibly show up as an increase in respiration (fig. 1 A and D). Recognizing the possibility that a factor other than sucrose might limit the respiration, O_2 uptake could not be relied upon as an accurate measure of the translocation of all concentrations of sucrose (or its hydrolytic products) to the cells in the root tips.

It is recognized that many interpretations might be offered for this effect of boron, including the possibility that it is directly affecting some phase of

the respiratory mechanism. In view of the fact that such a role has not been established, this possibility is minimized. An alternative possibility is that the enhanced respiration is effected by an increase in the rate of entry and movement of the substrate to the respiring cells in the root tips. The Warburg technique yielded indirect evidence supporting this concept.

In order to supplement the respiration studies, a tracer technique involving labeled sucrose was employed. This latter technique provided a direct

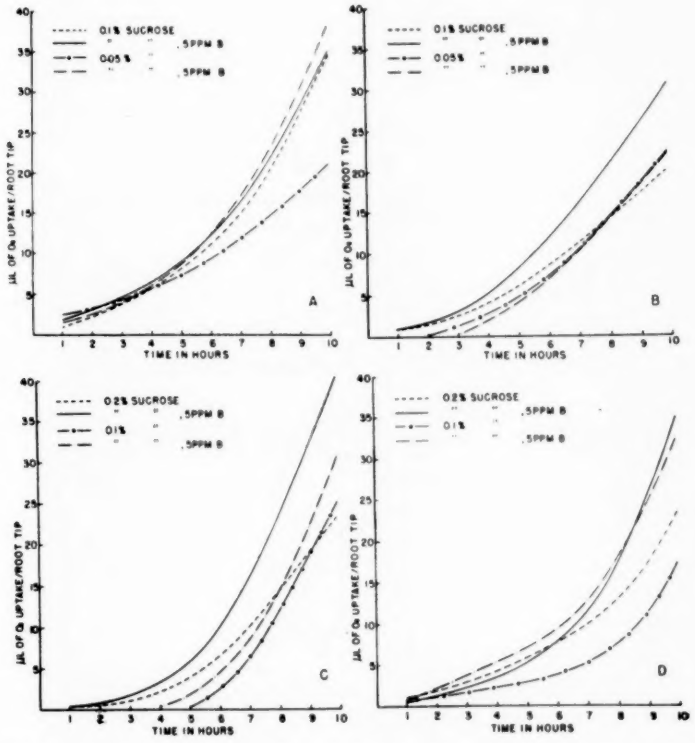


FIG. 1. The respiratory utilization of sucrose by root tips as affected by 5 p.p.m. of boron. Endogenous respiration of controls without added substrate has been subtracted. A, B: Five lima bean root tips per vessel; C, D: Eight pea root tips per vessel. All treatments were triplicated.

measure of the movement of sucrose (or its hydrolytic products) and, in contrast with the Warburg technique, did measure the full effect of boron on translocation. A single leaf of an intact plant (**24**) was immersed into a solution of labeled sucrose. After various intervals of treatment (4 to 24 hours) the plants were fractionated, the samples oven-dried, and the intensity of radioactivity in the various portions was determined. MITCHELL and LINDER'S (**17**) technique was employed for preparing the samples of dried and ground plant material prior to counting with a windowless flow counter.

With tomatoes which had been grown in sand under a low level of mineral nutrition receiving no boron and depleted of carbohydrate by a 48-hour period of darkness (**24**), the uptake of sucrose by an immersed leaf and its distribution throughout the plant were very rapid and striking when

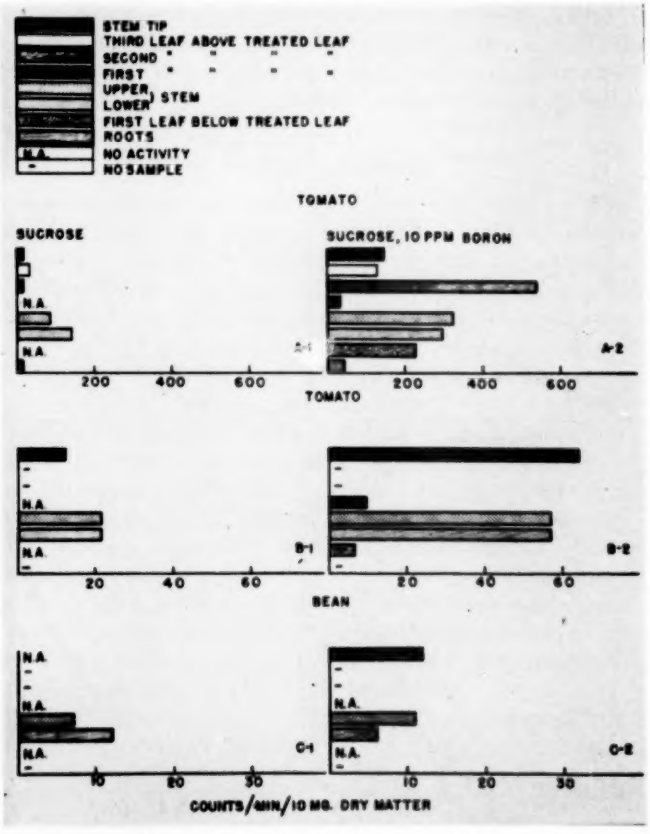


FIG. 2. The intensity of radioactivity (C^{14}) in various plant parts as a result of the movement of labeled sucrose (or its hydrolytic products) from a lower leaf immersed for 24 hours in a solution of labeled sucrose or labeled sucrose plus boron. All treatments were triplicated. A. Tomatoes cultured in sand, receiving no boron in the substrate, and subjected to 48-hour dark period prior to and during treatment; B. Soil-grown tomatoes receiving complete nutrition and kept in the light; C. Soil-grown snap bean plants receiving complete nutrition and subjected to a 48-hour dark period prior to and during treatment.

10 p.p.m. of boron was supplied with the sugar (fig. 2, A-2). In the absence of additional boron the sucrose entered in small amounts and only into the stem near the point of attachment of the immersed leaf (fig. 2, A-1). There was no directional mobilization of sucrose to the stem tip and to the young, developing leaves such as occurred in the presence of boron (fig. 2, A-2).

These data show the striking magnitude of difference between the translocation of sucrose (or its hydrolytic products) in plants deficient in boron and those adequately supplied with boron.

Under a deficiency of boron the root tips die sooner than the stem tips (**8**) even though the root tips are the first to come in contact with the boron that is available. However, if boron deficiency symptoms are caused by a deficiency of sugar, then it is understandable that the stem tips which are nearer the source of production of carbohydrates would be in a more favorable position and would live longer than the root tips. These observations are interpreted as evidence for the hypothesis that boron deficiency is actually a matter of sugar deficiency. With a low level of carbohydrate in the plant the tops have been reported by HOAGLAND (**12**) and others to utilize the available carbohydrate to the exclusion of the root system, resulting in higher top/root ratios.

In an experiment with vigorously growing, young greenhouse tomatoes the presence of 10 p.p.m. of boron in the sucrose solution enhanced the entry and movement of sucrose into the stem and stem tip (fig. 2, B-2 compared with B-1), even though the plants were receiving boron from the soil and they were not depleted of carbohydrate by a period of darkness. This is consistent with the hypothesis proposed herein. The enhanced movement of sucrose could conceivably proceed even beyond the capacity of the plant to utilize all of the sugar as fast as it is being translocated. EATON (**11**) observed that boron-toxicity symptoms and maximal growth overlapped in many of the species of plants which he studied.

In an experiment with snap bean plants grown in soil and adequately supplied with boron but depleted of carbohydrate by a 48-hour period of darkness, the effect of boron on the mobilization of sucrose by the stem tip is shown (fig. 2, C-2 compared with C-1).

Discussion

In a biochemical study of boron-deficient and sufficient plants, JOHNSTON and DORE (**13**) in 1928 reported that the leaves of boron-deficient plants have comparatively high concentrations of sugars and starch, whereas the concentration of sugars in the stems is low. Their data indicated that in the absence of adequate boron the translocation of sugars from the leaves to the stems is impaired, but the authors attributed this to a breakdown of the conducting tissues, particularly the phloem. ASKEW *et al.* (**3**) reported an increase in the per cent. of sugar in raspberry fruits by a soil application of boron to certain field-grown plants. Likewise, in a very exhaustive study on the Eastern Shore of Maryland, STARK and MATTHEWS (**22**) found that the per cent. of soluble solids (mostly sugar) in cantaloupe fruits could be increased from around 8% to higher than 10% by spraying the leaves with either borax or magnesium sulphate solutions. It would appear that increases in the concentration of sugars in fruits of plants supplied with supplemental boron must be the result of an increase in translocation of sugar rather than an effect on photosynthesis, since the leaves of boron-deficient plants are

consistently, abnormally high in sugars and starch. Inasmuch as SHEAR *et al.* (**21**) have discussed evidence that the boron level in plants may be affected by the magnesium level, it seems likely that the response to magnesium was an indirect effect so far as the translocation of sugar in cantaloupe is concerned (**22**), the causal relationship in translocation being between boron and sugar. The fact that the application of magnesium and boron in combination did not result in a greater effect than with boron alone would also indicate that the enhanced sugar translocation was strictly a boron effect. Calcium could also affect the translocation of sugar indirectly through its well-established relationship to the boron level in plants (**6**).

The hypothesis presented and substantiated by the experimental data, presented herein, would account for boron deficiency symptoms. It is almost universally reported that root and stem tips die and that flowers and fruits abort and abscise. These are all regions of high metabolic activity, therefore requiring large amounts of sugar to sustain their respiration and to permit their characteristically rapid rates of growth. WHITE-STEVENS (**25**) found that carbohydrates are deficient in the meristems and roots of boron-deficient plants, even though the leaves have an excess of carbohydrate in all forms. It would appear certain then that, in the absence of adequate boron, certain parts of the plant die owing to a lack of sugar. It is submitted that boron deficiency symptoms are an expression of sugar deficiency. WENT and CARTER (**24**) have shown by the spraying of sucrose solution on plants that the abscission of tomato flowers under high night temperatures is caused by an inadequacy of sugar, and that sugar (not auxin) is the limiting factor. Flowers also abscise under boron deficiency. In the light of WENT and CARTER's (**24**) findings and the research presented herein, an explanation is now available for the abortion and abscission of flowers associated with a deficiency of boron.

It is interesting to project this concept into a consideration of the formation and translocation of hormones. The observations by ALEXANDER (**2**) and CHANDLER (**8**) that boron-deficient plants lose their capacity to respond to gravity would indicate that there is a relationship between boron and either the production or the translocation of hormones, or both. With 2,4-dichlorophenoxyacetic acid applied to bean leaves, MITCHELL and BROWN (**16**) observed that the movement of the growth regulator was associated with the movement of carbohydrate from the treated leaves. BRENCHLEY and THORNTON (**5**) reported that in the absence of boron a vascular connection was poorly if at all established between the nodules of legumes and the vascular tissues of the root. This differentiation may be controlled by growth regulators which also normally move in association with sugar, but both of which would fail to move in the absence of adequate boron. The growth regulators may actuate and control the differentiation, and carbohydrate would certainly be required for the syntheses associated with differentiation (*e.g.*, cell wall thickening). It was not determined whether the increased fruit set of apparently healthy Anjou pear trees sprayed with boron (**4**) was caused by an increased translocation of sugar, growth regu-

lators, or both to the flower buds. However, BATJER and THOMPSON (**4**) report that Degman (unpublished work) could not increase the fruit set of Anjou pears by applying various growth-regulating chemicals. This latter work would indicate, as WENT and CARTER (**24**) maintained, that not auxin but sugar is the controlling factor. However, the research by MITCHELL and BROWN (**16**) which first showed the relationship between the movement of growth regulators and carbohydrates must be kept in mind when evaluating the results obtained by sucrose applications alone. Hormone assay would appear to be necessary for a reliable interpretation of such data. PILAND *et al.* (**19**) were able to increase the yields of alfalfa hay about 10% by an application of boron in the field while, at the same time, the yield of seed was enhanced by as much as 600%. SCOTT (**20**) reported an improved set of grapes by the application of boron at certain locations in the field even though the plants showed no evidence whatsoever of boron deficiency.

It is recognized that at least two possibilities exist for the manner in which boron accentuates the translocation of sucrose or its hydrolytic products. First, the borate ion could react with sucrose (or glucose or fructose), the sugar then passing through the cellular membranes as the ionized sugar-borate complex until such time as a cell utilizes this complex and liberates the borate ion. Secondly, it is possible that the borate ion is associated with the cellular membranes, that it there reacts chemically with the sugar molecule facilitating its passage through the membrane, and that the sugar is freed on the inside of the cell by a second reaction. In either event these two hypotheses are consistent with the well-established fact that at any given time in the life of the plant a small amount of boron suffices. However, plants must continue to absorb boron throughout their life cycle, indicating that, as new cell membranes are formed, more boron is required which would imply some degree of immobility of boron to new cells and tissues and, indeed, the evidence indicates that boron is relatively immobile in plants (**11**). The evidence thus favors the concept that boron is associated with the membranes.

The authors recognize that factors other than boron may be involved in the absorption and, perhaps, in the translocation of sucrose. DORMER and STREET (**9**) and STREET and LOWE (**23**) have postulated that sucrose is phosphorylated prior to its entry into tomato roots, and DOUDOROFF (**10**) has obtained from *Pseudomonas saccharophila* an extract containing a phosphorylase specific for sucrose. DORMER and STREET's (**9**) report concerning the rapid absorption of sucrose by tomato roots is in direct conflict with the report by WENT and CARTER (**24**) to the effect that sucrose does not enter through the roots of tomato plants. The extensive use of sucrose as a solute for inducing and maintaining plasmolysis would indicate that sucrose phosphorylase must indeed be of limited distribution in plant tissues, since the plasmolyzed cells do not recover.

DORMER and STREET (**9**) hypothesize a phosphorylation of sucrose at the surface of the protoplast, passage of the sugar through the membrane in the form of fructose-1-6-diphosphate, and a dephosphorylation reaction inside

the cell. Their findings concerning the non-permeability of tomato roots to simple sugars would thus appear to make mandatory a re-synthesis of sucrose prior to the movement of sugar into an adjoining cell. Although the phosphorylation of sucrose may be of importance prior to the entry of this sugar into certain cells or organs, the authors are inclined to the view, supported by evidence presented in this paper, that boron is the dominant factor in the movement of sucrose (or its hydrolytic products) from cell to cell within the plant. BROWN (7) has proposed that a transport mechanism is involved in the movement of sugar across a diffusion barrier such as a membrane, and it is hereby suggested that boron may be involved in or associated with the hypothetical reactive points in the membrane.

Summary

An hypothesis is presented and supported with experimental evidence to account for one essential role of boron in plants. The data suggest that one role involves a reaction of boron with sugar to form a sugar-borate complex (ionizable) which moves through cellular membranes more readily than non-borated, non-ionized sugar molecules.

Studies employing the Warburg apparatus were indicative of greater O_2 uptake by lima bean and pea root tips in the presence of sucrose plus 5 p.p.m. of boron, as compared with sucrose alone. A tentative interpretation of the increased (50 to 85%) respiration was that it was the result of an increase in the movement of sucrose (or its hydrolytic products) to the respiring cells. Five p.p.m. of boron had no effect on the endogenous respiration.

By employing uniformly labeled sucrose both alone and in the presence of 10 p.p.m. of boron, it was shown that there was a striking dependence of translocation of sugar on the presence of boron. When a leaf of a tomato plant was immersed in a labeled sucrose solution containing 10 p.p.m. of boron, the stem tip of the plant had 550% more radioactivity than if the solution contained no boron.

Data are presented which may be interpreted as evidence for the fact that sugar does not move readily through cellular membranes unless the sugar is borated. It is submitted, therefore, that boron deficiency symptoms are an expression of sugar deficiency in the cambia, stem tips, root tips, and flowers or fruits.

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CHANGES IN THE CARBOHYDRATE RESERVES OF LADINO WHITE CLOVER FOLLOWING DEFOLIATION[1]

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Defoliation of forage crops by cutting or grazing brings about a reduction in the weight and in the carbohydrate content of the remaining parts. The latter result has been studied by a number of workers in the grasses and in alfalfa, but with the few exceptions noted here it has not been studied in Ladino white clover, *Trifolium repens* L. TESAR and AHLGREN (**6**) concluded after a brief study that close and frequent cutting lowered the percentages of available carbohydrates in the stolons. STEWART and BEAR (**3**) showed that starch is an important storage product in the stolons and is utilized during the period of regeneration of new top growth. In this paper are reported results of studies to determine (a) the carbohydrate content of stolons and roots of Ladino clover and (b) fluctuations in carbohydrates during intercutting periods.

Experimental procedure and results

The stolons and roots of the plants to be analyzed were obtained either from pastures at State College, Pennsylvania, or from plants grown in soil in the greenhouse. Analyses were made by methods described elsewhere (**4**). The carbohydrates of stolon segments of different ages were determined in samples taken at three fall dates from a one-year-old pasture of Ladino clover and orchard grass which was ungrazed during the sampling period. The results are reported in table I. Sugars are on the dry-weight basis, but starch in this case only is reported as percentage of the alcohol-insoluble residue. Expressed as percentages of the dry weight, starch would be about one fourth lower but the relative differences would still hold. Starch was found to be the dominant carbohydrate and its highest concentrations were in the older parts of the stolons. Sucrose, varying from 3 to 14%, was also higher in the older than in the younger internodes. Reducing sugars were relatively low and consisted in greater part of glucose and in lesser part of fructose; both of these sugars were distributed rather uniformly without regard to segment age. Differences were also noted among sampling dates. The last sample, taken in November, was higher in sucrose (and total sugars) and lower in reducing sugars and starch than the earlier samples.

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TABLE I

DISTRIBUTION OF CARBOHYDRATES IN THREE SECTIONS OF STOLONS OF LADINO CLOVER PLANTS TAKEN FROM A PASTURE AT THREE FALL DATES.

Date	Section	Per cent. of dry weight			
		Starch*	Sucrose	Glucose	Fructose
October 17	First 5 internodes	19.48	3.21	3.06	0.61
	Second 5 internodes	32.08	6.21	3.84	0.70
	Older parts of stolon	33.05	6.47	2.96	0.61
October 22	First 5 internodes	19.05	2.97	2.75	0.58
	Second 5 internodes	26.24	6.31	3.48	0.60
	Older parts of stolon	34.49	7.73	2.34	0.65
November 21	First 5 internodes	18.39	10.48	2.14	0.50
	Second 5 internodes	16.79	14.59	1.49	0.61
	Older parts of stolon	21.65	13.50	1.13	0.50

*Sugars are in percentage of total dry weight and starch is in percentage of the alcohol-insoluble portion.

The younger parts of the stolons increased in total carbohydrates between October and November, but the older parts, because of the loss of starch, did not. Such comparisons indicate that changes took place during this part of the season and, since no defoliation occurred, the chief operating influences were seasonal.

Changes induced in the carbohydrate contents of the stolons by defoliation were determined in a number of experiments on greenhouse plants subjected to a variety of cutting treatments and environmental conditions. One experiment here reported included a mixed group of plants consisting of 15 clones, the clones representing a wide range of morphological types. Two levels of reserves were induced, one by making a single defoliation on May 4, 1950, and the other by making three defoliations within a period of 18 days, the last of which took place also on May 4. The composition of the plants on this date is given in table II. It may be seen that reducing sugars were higher and starch lower in these stolons than in those of plants from

TABLE II

THE CARBOHYDRATE CONTENT OF STOLONS AND ROOTS OF GREENHOUSE-GROWN LADINO CLOVER ON MAY 4.

Description	Part of plant	Per cent. of dry weight		
		Starch	Sucrose	Reducing sugars
High reserve plants previously cut only once				
	Stolons	17.67	3.81	5.58
	Roots	8.67	3.28	1.39
Low reserve plants previously cut three times				
	Stolons	3.80	1.40	0.60
	Roots	2.86	1.09	0.27

the pasture. Roots were lower than stolons in all the carbohydrate constituents. Those plants clipped three times were lower in all the constituents than those clipped once and, from the standpoint of composition, may be considered as low-reserve plants.

After these plants were defoliated new leaves were formed while the stolon and root weights and the carbohydrate composition changed. The changes were followed in the greenhouse in the light and also in a dark room. Plants were removed at intervals during the next three weeks for weighing and analysis. New leaf growth was most rapid in the high-reserve plants (clipped only once), which were allowed to remain in the greenhouse and by 21 days it amounted to 4.29 grams foliar dry matter per plant. The

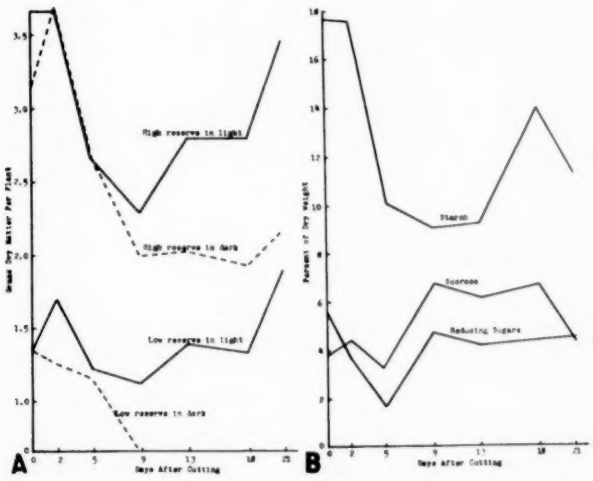

FIG. 1. A. Changes in the dry weight of the stolons of Ladino clover plants grown in the greenhouse; high- and low-reserve plants in light and in darkness. B. Changes in the several carbohydrates of the stolons of the Ladino clover plants, grown in the greenhouse, after defoliation; high-reserve plants in the light.

low-reserve plants grew more slowly and produced only 1.28 grams during the same period. Of the plants in the dark those with high reserves made rapid leaf growth at first but produced only 0.95 grams in 21 days while those with low reserves produced practically nothing.

The fluctuations that took place in the dry weights of the stolons are shown in figure 1 A. Losses in weight occurred for about nine days and were followed by increases in weight in those plants growing in light but not in those in darkness. The roots weighed about half as much as the corresponding stolons, and the changes produced in them were very similar to those in the stolons.

The carbohydrate contents of the stolons and roots changed after the defoliation. The greatest changes took place in the high-reserve stolons.

The several forms of carbohydrate decreased at first but later increased (figure 1 B), a process of utilization and restorage noted by others in grasses. Similar changes went on in the low-reserve stolons and in both sets of roots. For economy of space, only the changes in total carbohydrates (sugars plus starch) are illustrated in figure 2 A, but changes in the individual carbohydrates were parallel to those of the total. The high-reserve roots paralleled the high-reserve stolons. The low-reserve plants, perhaps because the reserves were already well depleted, showed a much smaller decrease in carbohydrates after defoliation, but they showed a definite tendency to rise later on. In all cases starch was restored more slowly than sucrose.

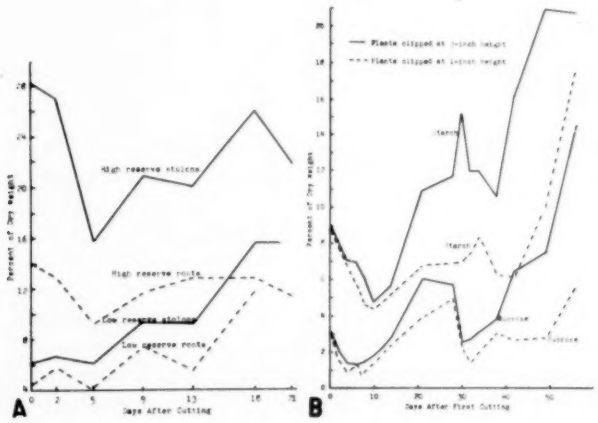

FIG. 2. A. Changes in the total carbohydrate content of the stolons and roots of Ladino clover plants grown in the greenhouse; of both high and low reserves; after defoliation. B. Changes in the starch and sucrose contents of stolons of Ladino clover clipped twice, 28 days apart, and at two heights, one inch and three inches above the soil.

Plants in darkness showed no restorage. While the high-reserve plants stayed alive during the whole 21-day period, they lost carbohydrates steadily. The low-reserve plants did not survive beyond nine days.

The effect of a second defoliation while the plants are recovering from the first defoliation was determined in another greenhouse experiment. When Ladino clover seedlings were about three and one half months old, they were defoliated by a clipping which also removed the ends of stolons extending over the sides of the pots. Some of these plants received a second defoliation at 12 days and others at 29 days after the first. Samples were taken at intervals for analysis after each cutting. Following the first cutting, the levels of all carbohydrate fractions decreased to reach minima at 10 to 12 days (fig. 3). The second cutting at 12 days caused further reductions. If the plants did not receive the second cut until the twenty-ninth day, the carbohydrates were by that time restored to a level comparable to that

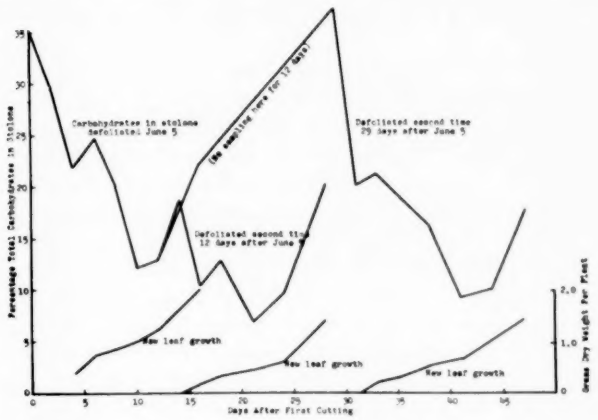
Carbohydrates in stolons defoliated June 5
(No sampling here for 12 days)
Defoliated second time 29 days after June 5
Defoliated second time 12 days after June 5
New leaf growth
New leaf growth
New leaf growth
Days After First Cutting

FIG. 3. Changes in percentages of total carbohydrates in stolons of Ladino clover defoliated twice, the first time on June 5, 1950, and the second time, some plants 12 days later and others 29 days later. Also, in the lower lines, increases in the dry weights of new foliage after each defoliation.

before the first cutting. A second cutting at this point again reduced stolon carbohydrates to reach a minimum at 12 days after the second cutting, a minimum which was intermediate to the other two. The greatest amount of new leaf growth was obtained after the first cutting and the least after the last cutting. The explanation of this is not obvious, but because this experiment carried over into the early summer season, it is probable that the greenhouse temperature eventually became higher than the optimum for Ladino clover. The composition of the roots paralleled that of the stolons

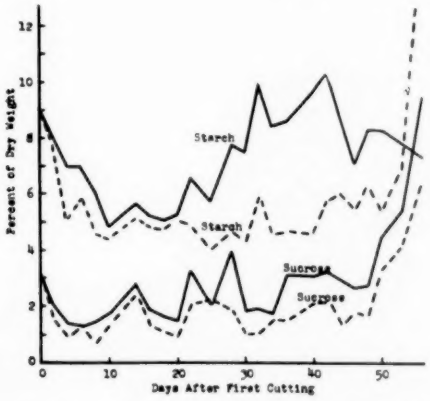

FIG. 4. Changes in starch and sucrose contents of stolons of Ladino clover clipped three times, 14 days apart, and at two heights, one inch and three inches above the soil. Plants clipped at three-inch height (——), plants clipped at one-inch height (– – –).

and the dry weight of both stolons and roots lost and regained to some extent after each cutting.

The effect of the height of cutting above ground level was investigated by comparing Ladino clover plants cut every 28 days at a height of one inch with others cut at a height of three inches. The three-inch cut permitted a number of small leaves on short petioles to remain, particularly near the center of the plant, but the one-inch cut removed most of these. Because of these remaining leaves the three-inch cut is considered to be not as severe a treatment as the one-inch cut. Starch and sucrose are markedly higher in the plants cut to three inches than in those cut to one inch (fig. 2 B). The typical curves of utilization and restorage are evident after the first cut and again after the second 28 days later. Other plants of the same group were cut more frequently, namely three times at 14-day intervals. In these plants it is again evident that the carbohydrates of plants cut at the three-inch level were higher than of those cut at one inch (fig. 4). A comparison

TABLE III

THE DRY WEIGHTS OF LEAVES (INCLUDING PETIOLES) OF GREENHOUSE-GROWN LADINO CLOVER CUT AT THE ONE-INCH AND AT THE THREE-INCH LEVELS AND AT 14 AND 28-DAY INTERVALS.

Days after first cut	Cut at one-inch level		Cut at three-inch level	
	14-day interval	28-day interval	14-day interval	28-day interval
	gm.	*gm.*	*gm.*	*gm.*
0	8.39	8.13	6.42	6.12
14	1.72	....	3.11	....
28	0.97	3.96	2.07	4.99
42	0.87	....	1.50	....

of figures 2 B and 4 will show that the plants cut more frequently are lower in carbohydrates. Those cut at one inch every 14 days are the lowest of all in reserve carbohydrates and as such do not exhibit the loss and restorage to such a degree as do plants with higher reserves. The effect of height of cut on the weight of leaves is evident (table III). At the first defoliation, cutting to one inch removed a greater quantity of leaves than cutting to three inches, but at each of the later dates the weight of new leaves removed was less with the one-inch cut.

Discussion

Carbohydrates appear to play a role as storage or reserve substances in Ladino clover in much the same way as they do in other forage plants. Starch, sucrose, and reducing sugars are to be found in both stolons and roots, more in the stolons than in the roots, but, considering that the roots are often of considerable size, the absolute quantity of carbohydrates in them may be appreciable. These carbohydrates undergo considerable fluctuation during plant growth. Some of these fluctuations are studied here.

Only a brief study was made of the carbohydrate content of plants in a pasture and only at one season of the year, the fall. In that short period it was noted that changes were occurring in the direction of lower starch and higher sucrose with the approach of colder weather. This is a trend that is noticed in many plants.

The reserve character of these carbohydrates, in relation to what might be called grazing management if carried on in the field, was studied on greenhouse plants. On their defoliation, or removal of the leaves to simulate grazing or mowing, the carbohydrates of the stolons decreased. This decrease continued for a week or two. By this time when new leaves were formed and producing new carbohydrates, the withdrawal from the stolons and roots ceased and increases occurred. It is assumed that this decrease and increase of carbohydrates from these parts meant their utilization and restorage. This utilization and restorage has been followed in both light and darkness and after defoliations of different intensities. The utilization was greatest when the carbohydrates were at a high level of storage, and under these conditions the amount of new leaves produced was highest. When the carbohydrates were at a low level, the typical curve of utilization and restorage was not so marked, and from this it appears that only those carbohydrates above a certain level act as reserves. The effects of a second defoliation, before the plant had recovered from the first, are illustrated here as well as those of successive defoliations at various frequencies and at various heights. Cutting at a low level reduced carbohydrates to a greater extent than at a higher level because it removed a larger number of leaves and hence delayed restorage. This restorage occurred only in light and continued as long as plant growth was undisturbed. In darkness, the stolons lost carbohydrates as rapidly as in light, but they continued to lose them for a longer time and did not show any restorage. When death resulted, some carbohydrates still remained but they were at a low level.

The amount of new leaf growth that was formed after cutting was related to the level of reserves. The high-reserve plants produced more than did the low-reserve plants and, in this experiment, those cut at a higher level from the ground produced more than those cut close to the ground. Under farm conditions, the level of reserves is dependent, among other things, upon two grazing management practices; namely, the frequency and the height of defoliation. In a pure stand of Ladino clover larger dry-matter yields are obtained over a period of time if the cuttings are made at a height of three inches above the soil rather than at a height of one inch. When Ladino clover and grass are growing together, however, the higher cutting has an opposite effect upon the clover in that it allows the grass to compete with the clover. Low cutting, on the other hand, removes more of the reserves of the grasses (**2, 5**) than it does from the Ladino clover and reduces their competitive effect (**1**). The most advantageous height and frequency of defoliation will be determined by the herbage and the conditions under which it is grown.

Summary

Starch and sugars were determined in stolons and roots of Ladino clover plants taken from pastures and from soil cultures in the greenhouse. Starch was found to be the main storage product and sucrose often exceeded reducing sugars. The concentrations of each were greater in stolons than in roots. Defoliation caused a depletion of these reserves from both stolons and roots. The depletion was greater when the level of the stored substances was high and not so great when the levels were lower. After a period of one or two weeks restorage occurred. The amount of stored carbohydrates was affected by the number and frequency of defoliations and by the height above the soil at which the cutting was made. The amount of new foliar growth produced after the defoliation was closely related to the level of carbohydrates at the time of the defoliation.

The authors gratefully acknowledge the technical assistance of Mrs. Patricia Altieri.

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BOLTING AND FLOWERING OF SUGAR BEETS IN CONTINUOUS DARKNESS

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Introduction

Since the discovery by GARNER and ALLARD (**3, 4**) that day length is one of the important factors in flowering, certain plants have been classified on the basis of their light requirement for flowering as short-day, intermediate, or long-day species. It is reported (**6**) that all varieties of *Beta vulgaris* investigated have been found to be long-day plants (**1, 4, 6**). STOUT (**8**) states that all beet varieties, however, require long photoperiods before they will bolt normally, and all varieties may be kept in a vegetative condition indefinitely by growing them in an environment of short photoperiods and warm temperature. It has been demonstrated repeatedly that sugar beet varieties require a prolonged low-temperature exposure before they will bolt. The induction of flowering of sugar beets, under the influence of prolonged low temperatures, has been designated as thermal induction. STOUT (**9**) studied the relation of temperature to reproduction in sugar beets and found that beets bolted more readily following a storage period at 6° C than after storage at lower temperatures for the same period of time. It was observed that storage near 0° C induced little change in the rate and percentage of bolting indicating that the processes involved in thermal induction are nearly arrested at this temperature. Reversal of the process was reported to occur in roots that had been thermally induced previously and then stored at 11 to 26° C.

It has been pointed out (**10**) that biennial beets require prolonged exposure to cool temperature (thermal induction) followed by, or concurrent with, exposures to long photoperiods for normal bolting and flowering to take place. Further investigation by Stout led to the conclusion that apparently either long day-length or thermal induction, acting independently, can carry the reproductive processes far toward completion and that the effects of light and low temperature exposure on reproduction in the sugar beet seem to be complementary. OWEN *et al.* (**6**) stated that induction of flowering under the influence of prolonged low temperature and long photoperiods may be tentatively regarded as a single process and may therefore be designated by a single term. The term photothermal induction was proposed by them to cover a broader meaning because, with beets, it was difficult in many cases to distinguish between these two environmental effects.

In 1945, the senior author, in connection with biochemical investigations, obtained evidence that if certain of the common sugar beet varieties, grown

for sugar production, receive sufficient thermal induction they will go into the reproductive phase, *i.e.*, bolt in continuous darkness. This suggested that for the common sugar beet varieties, thermal induction may play a much more important role than the photoperiod in initiating the reproductive phase in sugar beets. In view of these observations and the need for studying the thermal induction requirements of breeding stock used for developing new varieties of sugar beets, it appeared desirable to obtain further evidence on the relative importance of thermal induction and day length in relation to the initiation of the reproductive phase only. The importance of day length in bringing the fruiting phase to completion is clearly recognized.

Methods and results

A small interior room was prepared for growing beets in continuous darkness. The room, four by 12 feet, was darkened by painting the walls and ceiling black and by installing three sets of black drapes for added protection. A photographic film was used to test for presence of light. One third of the film was exposed for two weeks and the remainder for one week. The film was developed after the second week, and showed that it had not been affected by light.

For these studies three varieties of sugar beets, US 22/3, US 56/2, and US 75, were grown in well fertilized field plots. The roots were dug for all tests, except one, between July 20 and November 8, which was before they acquired a significant amount of thermol induction. The roots were washed, trimmed, and packed fresh in orange field boxes lined with two thicknesses of heavy brown waxed paper placed at right angles to each other. Moist pine shavings (90% saturation) were used as packing material (**2**). The packed boxes of roots were stacked in a dark room maintained at 40° F, which is considered to be near the optimum for thermal induction. Photographic films were packed in an orange field box in dry pine shavings, in the same manner as the roots, and placed in full sunlight for two hours, after which the films were recovered and developed. The results indicated that the boxes as packed were light-proof and that the roots went through the thermal induction period in complete darkness.

After various periods of thermal induction, boxes of roots were transferred from the thermal induction chamber to the dark room where they were unpacked, potted, and cared for in continued darkness. The temperature was maintained at 70° F during the period of growth in continuous darkness in all experiments except three. In these three tests the plants were grown at a temperature of 60 to 64° F in one experiment, in a second at 82° F and in the third the plants were grown 26 days at 65° F and then at 60° F during the remainder of the experiment.

Twelve experiments were conducted over a period of more than a year in which the three varieties of sugar beets were grown in continuous darkness immediately following a thermal induction period in complete unbroken

darkness. To serve as controls for each test conducted in continuous darkness, an equal number of roots, or more, were either replanted in the field or potted and placed in the greenhouse at a day length extended to 16 hours with the aid of a bank of mazda lamps. The results of the experiments conducted in continuous darkness are shown in table I.

Since all control plants bolted and flowered, the results from these plants are not included in the table. The plants growing in continuous darkness bolted sooner than did control plants in the greenhouse at a day length of 16 hours, and much sooner, but in no greater numbers, than control roots transplanted in the field. The more rapid bolting of plants in the dark was

TABLE I

BOLTING AND FLOWERING OF SUGAR BEETS GROWN IN CONTINUOUS DARKNESS FOLLOWING A THERMAL INDUCTION PERIOD IN COMPLETE DARKNESS.

Variety	Thermal induction period at 40° F.	Number of plants				
		Tested	Bolted	Producing flower buds	Producing open flowers	Reverting to vegetative phase
	Days					
US 56/2	100	12	12	0	0	12
"	214	3	3	2	1	2
"	230	10	10	2	2	8
US 22/3	138	8	8	1	1*	7
"	148	6	6	0	0	6
"	168	6	6	0	0	6
"	170	10	10	0	0	10
"	188	4	4	0	0	4
"	208	9	9	0	0	9
"	224	6	6	0	0	6
"	353	6	6	6	3	6
US 75	207	13	13	3	1	12

*34 days after potting, all seed stalks except two were removed with the aid of a one-watt fluorescent bulb covered with two layers of red cellophane wrapping tissue. Many well developed buds and many open flowers were found after 75 days.

no doubt due to the more constant favorable growing temperatures maintained in the dark room.

Seed stalks developed in complete darkness at temperatures ranging from the thermal induction temperature of 40 to 82° F. Flowering occurred from 64 to 82° F. At the higher temperature, many open flowers were found on plants examined after 25 days growth in continuous darkness, and open flowers were found on beets after 94 days of growth at the lower temperature. It appears that both terminal and axillary buds are influenced by the thermal induction treatment and as a result are capable of developing seed stalks in continuous darkness. As many as 47 seed stalks ranging in height from three to 36 inches were counted on one beet plant that flowered.

The plant shown in figure 1 is typical of the bolting and flowering that took place in continuous darkness. The pollen appeared to be normal in

size and color when compared with pollen collected from normal beets grown in the greenhouse at the same time.

Table I shows the number of roots in each experiment that bolted but reverted to the vegetative condition before flowering. After the initial rapid growth of seed stalks in continuous darkness, the rate of seed stalk elongation decreased slowly in these plants until the stalks produced vegetative tips. This decrease in rate of growth may have resulted from progressive depletion of reserves in the root. One root, weighing approximately 1500

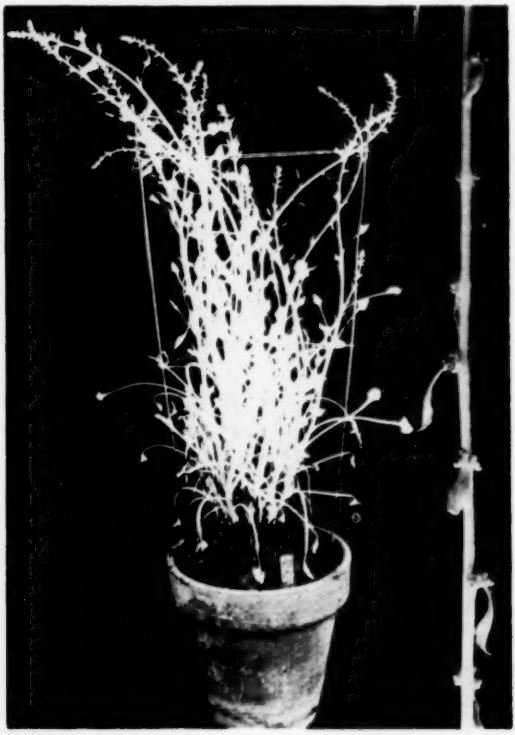

FIG. 1. Typical bolting and flowering of sugar beets grown in continuous darkness.

grams, produced nine seed stalks in continuous darkness averaging 54 inches in height, before reverting to a vegetative condition. The carbohydrate reserves were so depleted by this time that a sucrose determination on the root gave a saccharimeter reading of minus 1.3, indicating that all sucrose had been inverted. Other large roots, containing 14 to 16% sucrose initially, lost only 60 to 70% of their stored sucrose before they reverted to a vegetative condition. From this is appears that there may be other factors, in addition to a depletion of the carbohydrate reserves, which cause the plants to revert to a vegetative condition when grown in continuous darkness.

Most sugar beet roots, stored for 250 days or longer at 40° F in complete continuous darkness, produced seed stalks during the storage period. Many seed stalks were three to four inches long and some were six inches long. In one box containing 20 roots, which had received 270 days of thermal induction, all had bolted. This shows conclusively that, regardless of what may be the function of light, admittedly very important in carrying the process of fruiting to completion, it is not essential, either during or following the thermal induction period, to the initiation of seed stalk production.

There is strong evidence that if roots are given long periods of thermal induction, beyond that required for normal bolting and flowering, the subsequent day length requirement for their reproductive development under field conditions may be shorter. In Europe, MUNERATI (5) found that mother beets, after a storage period between 1 and 2° C, bolted and flowered during the winter months when transplanted in boxes in early September and placed outside. The boxes were covered with panes of glass to give protection against frost. The variety of beets used or the length of the thermal induction period was not reported. In one test conducted at Riverside, California, large roots (variety US 15) that had overwintered in a field plot were selected. During the previous winter the roots had received sufficient thermal induction for complete bolting and flowering. The roots were dug in the spring, before they had started to bolt, and stored in moist pine shavings at 40° F for an additional 188 days of thermal induction. The roots were transplanted in the field on September 28. The plants bolted by the middle of October and by the middle of December they had started to bloom. Normal seed was produced.

Discussion

Both low-temperature exposure and long days are essential for best flowering and fruiting of commercial varieties of sugar beet under field conditions. However, the relative importance and specific functioning of low temperature and light have not been determined fully. The results of experiments reported here indicate that light is not essential to the initiation of seed stalks. They show also that light is not essential to the flowering of sugar beet plants provided they have received sufficient thermal induction. However, the failure of many thermally induced plants grown in the dark to continue the fruiting process beyond a stage of seed stalk production clearly indicates the importance of photoperiod in bringing the fruiting phase to completion.

Since thermal induction alone can cause the production of sufficient flowering hormone to induce bolting and flowering in the sugar beet, the question naturally arises as to whether the increased flowering and seed production under long photoperiods result from the production of additional flowering hormone under the stimulus of light or whether light exerts an influence on fruiting in some other manner. Some of the evidence now available suggests that, in addition to the production of flowering hormones,

the long photoperiod may be important in enabling the plant to make the most efficient use of them.

Summary

Sugar beet roots developed seed stalks in complete unbroken darkness during a long thermal induction period at 40° F. Large roots bolted and flowered in continuous darkness following a long thermal induction period in complete darkness over a range of temperature from 60 to 82° F. Seed stalks were initiated as readily and in as great a number when grown in continuous darkness as on similar roots grown in a long day, but a much higher percentage of the beets grown in a long day flowered. The evidence shows that light, during or following a long thermal induction period, is not necessary for the initiation of seed stalks and that a long day is not an absolute essential to the flowering of sugar beets.

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COMPARATIVE STIMULATION OF PARTHENOCARPY IN THE TOMATO BY VARIOUS INDOLE COMPOUNDS[1]

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The stimulatory effect of indole compounds on fruit-setting of several horticultural crops (**7, 8, 18**) was soon established following the identification of indole-3-acetic acid as a plant growth hormone (**12**). Strangely enough, however, for the stimulation of parthenocarpy many related compounds, some analogs of indole-3-acetic acid, were found more effective (**7, 8, 11, 23, 25, 27, 32**). Meanwhile, extracts of corn kernels in the milk stage were described as having a fruit-setting potency much greater than could be accredited to indole-3-acetic acid alone (**9, 26**). Recently the fruit-setting factor derived from the ethanol extracts of immature corn kernels was characterized by REDEMANN *et al.* (**16**) as the ethyl ester of indole-3-acetic acid and was found to induce parthenocarpic fruit development in the tomato when applied in dilutions of 1 part in 10,000. Thus, the fruit-setting activity was multiplied almost 100-fold compared with the free acid or the salts of heteroauxin.

Recent studies concerned with the relation of chemical structure to activity, the relative instability of indole-3-acetic acid, the enhancement of activity by the addition of chlorine to the ring structure of certain growth substances, the role of growth substances in *in vitro* culture of tomato fruits (**14**) and finally, the scant attention directed toward halogenated or otherwise substituted indole compounds in biological activity testing suggested a more detailed evaluation of this chemical group of compounds with respect to the comparative induction of parthenocarpic fruit development in the tomato.

Methods

A known quantity of the compound was dissolved in anhydrous, peroxide-free ether and diluted to the desired concentration. An aliquot of this solution containing the required amount of the compound was added to a known quantity of anhydrous lanolin and stirred until solution was complete. The ether was removed by immersing the containers of the solution in a hot water bath. For higher dilutions the same procedure was followed with smaller aliquots.

Estimation of the fruit-setting activity of the various indole compounds consisted of applying 1, 0.1, 0.01, and 0.001% lanolin solutions of each chemical to the ovary of the tomato flower from which the stamens had been

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removed. From 15 to 20 milligrams of lanolin containing the desired chemical were applied to each ovary. About 12 blossoms from the first flower clusters of three to four tomato plants (variety, Michigan State Forcing) of comparable physiological and nutritional status were employed for each dilution. The dilutions which caused definite fruit development of the emasculated blossoms were determined. The relative effectiveness of a particular substance at the various concentrations is indicated (table I) by the number of plus signs allotted. Three plus signs signify high activity with 100% set and fruit development equivalent or superior to natural pollination, two signs moderate activity with about 50% fruit set but with fruit size somewhat less than from pollination, and one plus sign slight but definite growth apparent in 10 to 25% of the ovaries treated.

The following compounds were obtained commercially: Indole-3-acetic acid, β(indole-3)-*n*-proprionic acid, γ(indole-3)-*n*-butyric acid, indole, isatin, L-tryptophan, and tryptamine from the Eastman Kodak Company, 3-methyl indole from the Matheson Company, and 2,3-diphenylindole from the B. F. Goodrich Chemical Company. The 4-chloroindole-3-acetic, 5-chloroindole-3-acetic, 6-chloroindole-3-acetic and 7-chloroindole-3-acetic acids were supplied by Dr. Sidney W. Fox of Iowa State College at Ames, Iowa. The 1-benzoylindole-3-acetic and 2-phenylindole-3-acetic acids were supplied by Mr. W. F. Houff of Michigan State College. The following compounds were prepared according to procedures given in the literature: methylindole-3-acetate, ethylindole-3-acetate, *n*-propylindole-3-acetate, *n*-butylindole-3-indoleacetate, *n*-amylindole-3-acetate (**17**), ethyl-5-chloroindole-3-acetate, 5,7-dichloroindole-3-acetic acid (**6**), 4,7-dichloro-2-methylindole-3-acetic acid (**10**), indole-3-pyruvic acid (**3**), indole-3-ethanol (**20**), 2-methylindole-3-acetic acid (**21**), 2-methylindole (**5**), indole-3-aldehyde (**19**), oxindole (**22**), and indole-3-carboxylic acid (**28**).

Results

The comparative effectiveness of various indole compounds at different concentrations to induce parthenocarpy in the tomato is given in table I. The methyl and ethyl esters of indole-3-acetic acid were approximately 100 times more effective than indole-3-acetic acid and were the only compounds tested having activity at a solution concentration of 0.001% in lanolin. The activity of the esters decreased as the size of the alkyl radical was increased with the exception of the methyl and ethyl esters which had identical activity. The introduction of chlorine on the benzene ring of indole-3-acetic acid in either the 4, 5, 6, or 7 position enhanced the activity 10 times above that of the free acid. However, the chloroindole-3-acetic acids were decidedly effective only at dilutions of 0.01%, and thus, were only one tenth as effective as ethylindole-3-acetate. Esterification of the chloroindole-3-acetic acids did not increase the activity to induce parthenocarpy as it did in the case of indole-3-acetic acid. The position of the chlorine in the benzene ring had no effect as far as fruit-setting effectiveness was concerned.

However, the addition of two chlorine atoms on the ring (5,7-dichloroindole-3-acetic acid) slightly reduced rather than enhanced the activity. Some effects on the fruit-setting response obtained by lengthening the alkyl radical of the indole acids has already been reported (**7, 8, 11, 23**).

Indole-3-pyruvic acid, indole-3-ethanol, and indole-3-nitrile induced fruit-setting only at 1% solutions. The introduction of a benzoyl radical

TABLE I

RELATIVE EFFECTIVENESS OF INDOLE COMPOUNDS IN LANOLIN ON THE STIMULATION OF PARTHENOCARPY IN THE TOMATO.

Compounds	Concentration of solutions			
	1.0%	0.1%	0.01%	0.001%
Indole-3-acetic acid	+++*	+++	+	Inactive
1-Benzoylindole-3-acetic acid	+++	Inactive	Inactive	Inactive
2-Methylindole-3-acetic acid	+++	Inactive	Inactive	Inactive
2-Phenylindole-3-acetic acid	+	Inactive	Inactive	Inactive
B-(Indole-3)-*n*-propionic acid	+++	Inactive	Inactive	Inactive
γ-(Indole-3)-*n*-butyric acid	+++	+++	++	Inactive
Indole-3-pyruvic acid	+++	Inactive	Inactive	Inactive
Methylindole-3-acetate	+++	+++	+++	++
Ethylindole-3-acetate	+++	+++	+++	++
n-Propylindole-3-acetate	+++	+++	+++	Inactive
n-Butylindole-3-acetate	+++	+++	++	Inactive
n-Amylindole-3-acetate	+++	+++	Inactive	Inactive
4-Chloroindole-3-acetic acid	+++	+++	+++	Inactive
5-Chloroindole-3-acetic acid	+++	+++	+++	Inactive
6-Chloroindole-3-acetic acid	+++	+++	+++	Inactive
7-Chloroindole-3-acetic acid	+++	+++	+++	Inactive
Ethyl-5-chloroindole-3-acetate	+++	+++	+++	Inactive
5,7-Dichloroindole-3-acetic acid	+++	+++	+	Inactive
4,7-Dichloro-2-methylindole-3-acetic acid	Inactive	Inactive	Inactive	Inactive
Indole-3-ethanol	+++	Inactive	Inactive	Inactive
Indole-3-nitrile	+	Inactive	Inactive	Inactive
Isatin	Inactive	Inactive	Inactive	Inactive
L-Tryptophan	Inactive	Inactive	Inactive	Inactive
Indole-3-aldehyde	Inactive	Inactive	Inactive	Inactive
Oxindole	Inactive	Inactive	Inactive	Inactive
2,3-Diphenylindole	Inactive	Inactive	Inactive	Inactive
Tryptamine	Inactive	Inactive	Inactive	Inactive

*The number of plus signs at each concentration indicates the relative magnitude of activity of each substance.

in position 1 or a methyl group on position 2 of indole-3-acetic acid reduced the fruit-setting activity to approximately one twentieth of the free acid. With the substitution of a phenyl radical in the 2 position of the indole nucleus, the activity was less than that of the 2-methylindole-3-acetic acid. This indicates that the 2 position must be left unsubstituted to obtain maximum efficiency. The introduction of a chlorine atom in position 4 as in 4,7-dichloro-2-methylindole-3-acetic acid destroyed the activity completely. This demonstrates that additional substitution on the benzene ring of

2-methylindole-3-acetic and especially in position 4 (ortho) eliminated entirely the fruit-setting activity of the compound.

Indole, isatin, 2-methylindole, 3-methylindole, L-tryptophan, indole-3-aldehyde, oxindole, 2,3-diphenylindole, indole-3-carboxylic acid, and tryptamine were inactive.

Discussion

The indole compounds occupy a unique position in that the only naturally occurring growth substance, the existence and identity of which is beyond dispute, is indole-3-acetic acid (heteroauxin) or its ethyl ester (**16**). It may now be safely assumed that indole-3-acetic acid is common to all green plants. In view of this, it is a striking fact that halogenated and otherwise substituted indole compounds have received only cursory attention; yet, highly active substances might logically be found among them.

In the present studies at least 10 indole compounds, specifically esters and chlorinated derivatives of indole-3-acetic acid, were found more effective than indole-3-acetic acid itself for stimulating parthenocarpic fruit development in the tomato. That a number of derivatives of indole-3-acetic acid, particularly the esters, may be more active than the unsubstituted acid in some plant growth responses has already been reported (**1, 29, 30, 31**), conversely, considerable evidence has been accumulated by the *Avena* and other biological assays that all substitutions so far studied do not increase and may greatly reduce the activity of indole-3-acetic acid (**2, 4, 13, 15, 24**). The present work on the enhancement of tomato parthenocarpic induction by chlorine substitution and esterification of indole-3-acetic acid interestingly demonstrates a parallel with some of the phenoxy acids, and the need for thorough testing by a number of assay methods including intact plants such as the tomato before evaluating the relative activity of a plant growth-regulator. It is conceivable that derivatives of even greater activity from the standpoint of fruit set might exist among the indole compounds.

The ortho effect in plant growth-regulators as reported by HANSCH and MUIR (**10**) and criticized by ZIMMERMAN and HITCHCOCK (**33**) and THIMANN (**25**) was observed in the fruit-setting responses induced by variously substituted indole compounds. Substitutions of the methyl or phenyl radical in the 2 position greatly reduced the activity, and substituents in both ortho positions as in 4,7-dichloro-2-methylindole-3-acetic acid completely eliminated activity, although 4-chloroindole-3-acetic acid was found more active than the parent compound. All fruit-setting responses could not, however, be explained by the ortho effect theory an example being the weak activity obtained with 1-benzoylindole-3-acetic acid.

Summary

In the tomato parthenocarpic fruit test, 10 derivatives of indole-3-acetic acid were found to have greater fruit-setting activity than the parent compound. Esterification of the carboxyl group of indole-3-acetic acid with a

methyl or ethyl radical increased the activity 100-fold. The activity of the esters decreased as the size of the alkyl radical was increased. Mono-substitution of the hydrogen by chlorine in any of the positions in the benzene ring of indole-3-acetic acid increased the activity 10-fold, and resulted in indole compounds one tenth as effective as methyl or ethylindole-3-acetate. In contrast to indole-3-acetic acid, esterification of the chloroindole-3-acetic acids did not increase the fruit-setting activity. The 1-benzoylindole-3-acetic acid and 2-methylindole-3-acetic acid were only one tenth as effective as the parent compound. Substitution of a phenyl group for a methyl radical in the 2 position further decreased the activity, and substituents in both positions ortho to the alkyl side chain completely eliminated activity.

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IRON AND COPPER ENZYMES IN LEAF LAMINA OF TOBACCO WHEN DEFICIENT IN MICRONUTRIENTS OR GROWN ON CALCAREOUS AND ORGANIC SOILS

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Certain enzyme responses in plants were considered by BAILEY and MCHARGUE (**1**), MACVICAR and BURRIS (**6**), and QUILAN-WATSON (**7**) as the effects of an element on a particular enzyme system. Enzymatic activity has been used by BROWN and HENDRICKS (**2**) as indicative of the type deficiency produced in chlorosis susceptible plants grown on a naturally calcareous soil and a copper-deficient organic soil. Relative activities of ascorbic acid oxidase (copper enzyme—AAO), catalase and peroxidase (iron enzymes) were determined for wheat, corn, tobacco, three species of lupines, and two varieties of soybeans. The ascorbic acid oxidase activity was reduced in plants grown on the copper-deficient soil and a comparatively reduced catalase activity was observed in plants grown on the naturally calcareous soil. These data verified the assumption that if copper or iron, respectively, were the limiting factors in the plant's nutrition, the deficiencies would be revealed as diminished activities of one or more of the enzymes requiring a specific element to function properly.

In recognition of the possibility that similar results might have been obtained through uncontrolled variations in other micronutrient elements, these experiments have been repeated, using tobacco, for the comparison of plants deficient in specific micronutrients. Comparisons of enzymatic activity patterns resulting from specific micronutrient deficiencies with those of plants grown on calcareous and organic soils were considered desirable. Coincidence in responses under the widely different environmental conditions of soil and water-culture would be strong additional evidence of the cause of the soil abnormalities. Moreover, since the calcium carbonate method for removal of micronutrient impurities also removes many of the unessential trace element impurities, the latter could also be minimized as contributory factors.

Methods and materials

Connecticut Broadleaf tobacco was grown in two-gallon crocks in the greenhouse on the following soil mixtures: (a) calcareous, pH 7.8; (b) two thirds calcareous and one third organic, pH 7.5; (c) one third calcareous and two thirds organic, pH 7.2; (d) organic, pH 6.0; (e) organic soil with added copper sulphate. The calcareous soil was a Millville silt loam obtained from Dr. D. W. Thorne of the Utah State Agricultural College, Logan, Utah. The copper-deficient organic soil was obtained from Dr. J. F. Davis and R. Gillespie of the Michigan State College Experimental Farm,

East Lansing, Michigan. The soils were mixed and fertilized as described previously (**2**).

Connecticut Broadleaf tobacco was grown at the same time in triplicate on solutions (10 liters) purified with a modified calcium carbonate method for removal of trace element impurities. Subsequent reports will appear on efficiency of purification treatment and on growth, reproduction, deficiency symptoms, and mineral absorption of the plants. The single-charge technique and type of equipment have been previously described (**10, 11**). Iron-, zinc-, copper-, manganese-, boron-, and molybdenum-deficient plants developed when grown on the solution cultures deficient in the respective element. Three separate experiments were conducted in which plants were grown both on the soils and solution cultures. Plants were grown in the spring and fall of 1951 and the spring of 1952. Deficiency symptoms developed by the plants prior or subsequent to sampling ranged from severe (Fe, Zn, Mn, B) to moderate (Cu, Mo). Plants suffering from severe deficiencies gave sharply reduced yields.

Samples of leaves for enzyme activity determinations were taken at intervals of about a week, beginning at an early stage of growth and ending at the flowering stage. Leaves similarly located in the middle of the plant, usually free from chlorosis and necroses, were selected for analysis. Three harvests were made during the growth of each plant and successive samples were often taken from different plants. In cases of severe deficiency a composite sample from three replicates was employed if necessary. Leaves from the plants were all harvested at the same time and placed in a refrigerator. The midribs of the leaves were then removed and the leaf lamina for each treatment combined and cut into small pieces with scissors. A ten-gram and a one-gram sample were weighed out for AAO and peroxidase activity determinations respectively. These samples were then placed in the freezing compartment of a refrigerator and AAO and peroxidase measurements made the day following harvest. Catalase activity was determined in a one-gram sample immediately after harvest. All the samples were taken early in the morning but no attention was given to light conditions at the time of harvest.

Catalase was determined by the gasometric procedure described by Bailey and McHargue (**1**) except that the tissue was macerated in a blender for two and one half minutes with ice present. Catalase activates the reduction of hydrogen peroxide into water and molecular oxygen. The rate of oxygen evolved was measured every minute for five minutes.

Peroxidase activity was determined by the method of James and Cragg (**4**) in which the rate of color change was measured; a brown color being produced by the oxidation of the substrate catechol in the presence of hydrogen peroxide. The peroxidases operate by splitting the peroxide into water and active oxygen, the latter combining with the catechol. Readings were taken in a Coleman spectrophotometer every 10 seconds for two and one half minutes.

Ascorbic acid oxidase activity was measured manometrically by determining the oxygen consumed in the enzymatic oxidation of the substrate

TABLE I

COMPARATIVE ENZYME ACTIVITIES FOR TOBACCO GROWN ON FE, CU, B, MN, MO AND ZN DEFICIENT NUTRIENT CULTURES (DATA EXPRESSED IN ARBITRARY UNITS).

Date	Days after transplanting	20 p.p.m. Fe	15 p.p.m. Fe	5 p.p.m. Fe	1 p.p.m. Fe	.5 p.p.m. Fe	.2 p.p.m. Fe	−Cu	−Cu*	−B	−B*	−Mn*	−Mo*	−Zn*
						Catalase								
11-30-51	28	244	....	....	451	....	86*	536	....	324	74	228	130	197
12- 7-51	35	350	....	....	319	....	51*	489	....	246	45	178	106	42
12-17-51	45	164	....	....	115*	....	....	470	380	....	33	145	115	61
4-14-52	34	....	440	475	....	....	....	....	404	434	....	....	203	....
4-21-52	41	....	274	128*	....	316	....	....	346	....	41	84	155	155
4-28-52	48	....	100*	....	....	75*	....	....	....	....	....	....	55	....
						Peroxidase								
11-30-51	28	28	....	....	24	....	11*	34	....	21	54	12	6	30
12- 7-51	35	41	....	....	35	....	26*	32	....	42	75	43	30	58
12-17-51	45	53	....	....	48*	....	....	57	66	....	81	83	33	93
4-21-52	41	....	47	41*	....	35	....	....	60	....	74	90	53	71
						Ascorbic Acid Oxidase								
11-30-51	28	172	....	....	129	....	149*	76	....	91	137	92	67	235
12- 7-51	35	119	....	....	142	....	119*	9	....	79	98	68	58	229
12-17-51	45	151	....	....	163*	....		13	12	....	216	76	72	268
4-14-52	34	....	203	224	....	162		....	10	....	105	....	40	....
4-21-52	41	....	244	284*	....	....		....	23	....	257	125	94	212

*Chlorosis or other deficiency symptoms evident.

L-ascorbic acid to dehydro-ascorbic acid. The method used was essentially that outlined by JAMES and CRAGG (**4**), except that the tissue sample was reduced to 10 gm., L-ascorbic acid substrate concentration was increased to 4 mg./ml. and air was used in the manometric flasks.

Comparative rather than quantitative extractions of the enzymes from the plant tissue were considered satisfactory to the objectives of this investigation. The plant sample was taken through two extractions with no precautions taken to obtain entirely all of the enzyme. Several duplicated experiments showed that the error between enzymatic extractions was about 15%. The consistency of the data obtained from the extraction procedures appeared to warrant comparisons of like plant material grown on the varied treatments. The AAO activity in plants grown on treatments which had received copper was approximately 10 times greater than in those which were copper deficient. The comparative AAO, catalase, and peroxidase activities were determined three times during the growth of the plants in three separate experiments making a total of nine determinations on each treatment during the course of this investigation. At each harvest the sampling and determinations were carried out under like conditions.

In the solution cultures slight experimental modifications in purification, treatment and handling of seedlings in successive experiments, resulted in some variations in degree of deficiency symptoms. Such changes were generally in the direction of intensified micronutrient deficiency effects, except for copper deficiency in one fall experiment. Some of these plants, however, showed leaf necroses and decreased yields on withholding copper to a greater degree than plants grown on the copper-deficient organic soil. Supply of minerals was ample for plants grown in the fall, but controls grown in the spring in the solution cultures sometimes displayed slight symptoms of nitrogen and iron deficiencies after flowering.

Results and discussion

The relative AAO, catalase, and peroxidase enzyme activity pattern for iron, copper, manganese, and molybdenum deficiencies seemed to be characteristic for each deficiency (tables I and IV). The boron and zinc patterns were similar but differed from those of the other micronutrients. Catalase activity decreased with deficiencies in iron, zinc, boron, and molybdenum; and increased only in the early stages of copper deficiency. Peroxidase activity remained about the same or decreased with deficiencies in iron and molybdenum; and increased in later stages of deficiencies in zinc, manganese, and boron. Ascorbic acid oxidase activity decreased in the plant with deficiencies in copper, manganese, and molybdenum. Copper-deficient plants had a much lower AAO activity than plants grown on the other treatments.

With the appearance of boron deficiency symptoms in tobacco, *i.e.*, blackening of the terminal bud, a marked and rapid enzymatic change occurred. Peroxidase and AAO activities were increased and catalase activity decreased. REED (**8**), MACVICAR and BURRIS (**6**), and KLEIN (**5**) observed an increase in polyphenoloxidase activity with the occurrence of

boron deficiency in plants. REED (9) also found a higher polyphenoloxidase activity in zinc-deficient than in normal plants.

The results obtained with the calcareous and organic soils are shown in table II. Catalase activity was lowest in the leaves of the chlorotic plants grown on the calcareous soil. Tobacco grown in the spring on these soils showed no symptoms of lime-induced chlorosis, gave higher yields than the fall plants (table III), and had a lower ascorbic acid oxidase activity. Though symptoms of copper deficiency were slight, or entirely absent, the copper-containing enzyme, AAO, was present in appreciably lower activity in leaves of plants grown on the organic soil.

TABLE II

COMPARATIVE ENZYME ACTIVITIES FOR TOBACCO GROWN ON CALCAREOUS, ORGANIC, AND ORGANIC-CALCAREOUS SOIL MIXTURES (DATA EXPRESSED IN ARBITRARY UNITS).

Date	Days after transplanting	Soils				
		1 Calcareous	2 Two thirds calcareous one third organic	3 One third calcareous two thirds organic	4 Organic	5 Organic + Cu
		Catalase				
12-12-51	40	210	462	441	480	389
12-19-51	47	212	350	349	425	308
12-31-51	59	188*	412	485	550	267
4-23-52	43	228	293	325	239	157
5- 6-52	56	192	208	212	190	103
		Peroxidase				
12-12-51	40	30	30	34	37	32
12-19-51	47	24	34	36	29	28
12-31-51	59	33*	45	58	50	59
5- 6-52	56	38	56	43	43	37
		Ascorbic Acid Oxidase				
12-12-51	40	237	217	89	12	222
12-19-51	47	279	250	115	8	205
12-31-51	59	321*	313	79	6	303
4-23-52	43	50	46	24	− 1	134
4-28-52	48	160	246	31	10	313
5- 6-52	56	143	62	24	27	213

*Chlorosis evident.

The results of enzymatic activities in plants grown on both solutions and soils have been summarized in table IV. The water-culture controls contained 15 and 20 p.p.m. Fe (table I). The soil control was the organic soil to which copper had been added (table II). A marked degree of specificity was indicated by responses in enzymatic activities to micronutrient deficiencies even though only three enzymes were studied. Several of these patterns were established prior to the formation of visual symptoms of deficiency. Note also that the enzymatic patterns for plants grown on the calcareous and organic soils were similar, respectively, with those of iron- and copper-deficient plants grown on water-culture. Such data are also in agreement with enzymatic measurements obtained with lupines, corn, and

TABLE III

YIELD OF TOBACCO GROWN IN THE FALL AND SPRING ON A CALCAREOUS, ORGANIC, AND ORGANIC-CALCAREOUS SOIL MIXTURE.

Date	Days after transplanting	Soils				
		1 Calcareous	2 Two thirds calcareous one third organic	3 One third calcareous two thirds organic	4 Organic	5 Organic + Cu
		gm. green wt./plant				
12-12-51	40	121	141	146	187	182
12-19-51	47	197	234	306	259	298
12-31-51	59	350*	547	607	521	528
5- 6-52	56	746	754	855	851	885

*Chlorosis evident.

the PI-54619-5-1 variety of soybean (**2**), which are all chlorosis-susceptible plants when grown on the calcareous soil. These data reflect the marked changes in these enzyme systems with varied nutrition and the range in adjustment of the tobacco plant to its environment.

ILJIN (**3**) made analyses of expressed sap from several plant species and found that ample iron was present in the sap of both normal and chlorotic plants. He concluded that chlorosis was due to the internal inactivation of iron. The results of this investigation are interpreted as indicating that the lime-induced chlorosis of plants grown on the calcareous soil is probably caused by iron deficiency: due either to internal inactivation or lack of iron in the plant. Also, the AAO enzyme activity in the plants studied appeared to be indicative of the external copper supply.

Summary

In determinations of comparative enzymatic activities for ascorbic acid oxidase, catalase, and peroxidase in Connecticut Broadleaf tobacco deficient in specific micronutrients (water cultures), each type of deficiency exhibited a characteristic enzymatic pattern of the leaf lamina. Tobacco grown on a calcareous soil (lime-induced chlorosis) gave the same type of enzymatic

TABLE IV

PATTERNS OF RELATIVE ENZYMATIC ACTIVITIES IN LEAF LAMINA OF CONNECTICUT BROADLEAF TOBACCO WITH MICRONUTRIENT DEFICIENCIES IN WATER-CULTURE; OR GROWN ON CALCAREOUS OR ORGANIC SOILS. INCREASE (+), DECREASE (−), NO CHANGE (0) RELATIVE TO CONTROLS.

Deficiencies	Catalase	Peroxidase	Ascorbic acid oxidase
Fe	−	0 to −	0
Zn	−	+	0
Cu	+	0	−
Mn	0 to −	0 to +	−
B	−	+	0
Mo	−	0	−
Calcareous soil (Fe)	−	0 to −	0
Organic soil (Cu)	+	0	−

pattern as on iron-deficient nutrient solutions. Likewise, plants grown on the copper-deficient organic soil gave the same type of enzymatic pattern as those grown on copper-deficient nutrient solutions.

Iron-deficient plants had a relatively lower peroxidase activity than boron- and zinc-deficient plants. Catalase activity was comparatively low in all three of these deficiencies.

Manganese-, molybdenum-, and copper-deficient plants showed a comparative decrease in ascorbic acid oxidase activity. The decrease was greatest in the copper deficient plants which had the highest catalase activity. Peroxidase activity appeared to be much higher in the manganese-deficient plants than in either of the other two deficiencies.

Some of the enzymatic patterns characteristic of the individual micronutrient deficiencies appeared in the leaves prior to or in the absence of the formation of visual symptoms.

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THE EFFECT OF THE DOMINANCE OF A METABOLIC SYSTEM REQUIRING IRON OR COPPER ON THE DEVELOPMENT OF LIME-INDUCED CHLOROSIS

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In an earlier experiment Brown and Hendricks (**1**) grew both chlorosis-susceptible plants and plants not susceptible to chlorosis on a calcareous soil of limited iron-supplying capacity, an organic soil of limited copper-supplying capacity and mixtures of the two soils. Since it was believed that a deficiency of either element in the plant would result in a changed activity of some enzyme, comparative enzymatic activities of ascorbic acid oxidase (a copper enzyme), catalase, and peroxidase (iron enzymes) were determined. Three varieties of lupines and one of soybeans developed a severe lime-induced chlorosis but showed no response to copper. These plants all exhibited comparatively low catalase activity. Lime-induced chlorosis did not develop in Thatcher wheat grown on the naturally calcareous soil, but the crop did show copper-deficiency symptoms when grown on the organic soil. Ascorbic acid oxidase activity was high in normal Thatcher wheat, and since this is believed to be the terminal oxidase in wheat (**7**), it may be a factor which allows the plant to grow on a soil with limited iron supplying capacity.

In the present experiment it was considered desirable to extend the above observations to additional crops to determine the extent to which this generalization might hold. A classification of plants seemed possible based upon the plant's response to the two types of nutrition studied.

Methods and materials

Thatcher spring wheat, Hannchen barley, Long Green okra, cocklebur, Ottawa Select spring rye, Red kidney beans, Marglobe tomatoes, and Connecticut broadleaf tobacco were grown on the following soils: soil 1, naturally calcareous, pH 7.8; soil 2, two thirds calcareous and one third organic, pH 7.5; soil 3, one third calcareous and two thirds organic, pH 7.2; soil 4, organic soil, pH 6.0; and soil 5, organic with added $CuSO_4 \cdot 5\,H_2O$. These soils provided a range of availability of both iron and copper. The plants were grown in the greenhouse on the above soil treatments in two-gallon glazed crocks as previously described (**1**).

Comparative measurements of the apparent activity of the enzymes ascorbic acid oxidase, catalase, and peroxidase were carried out under fixed procedures previously described (**1**). Ascorbic acid oxidase activity was determined manometrically and catalase gasometrically, using ascorbic acid and hydrogen peroxide, respectively, as substrates. Peroxidase activity was determined colorimetrically using catechol. All plant material was harvested

for analyses about 8:00 A.M. The leaves were taken from the upper half of the plant, combined, and cut into small pieces with scissors. Samples for ascorbic acid oxidase and peroxidase measurements were frozen overnight and determined the day following harvest. Catalase measurements were completed approximately two hours after harvest.

Comparative rather than quantitative extraction of the enzymes from the plant was considered satisfactory to the objectives of this investigation.

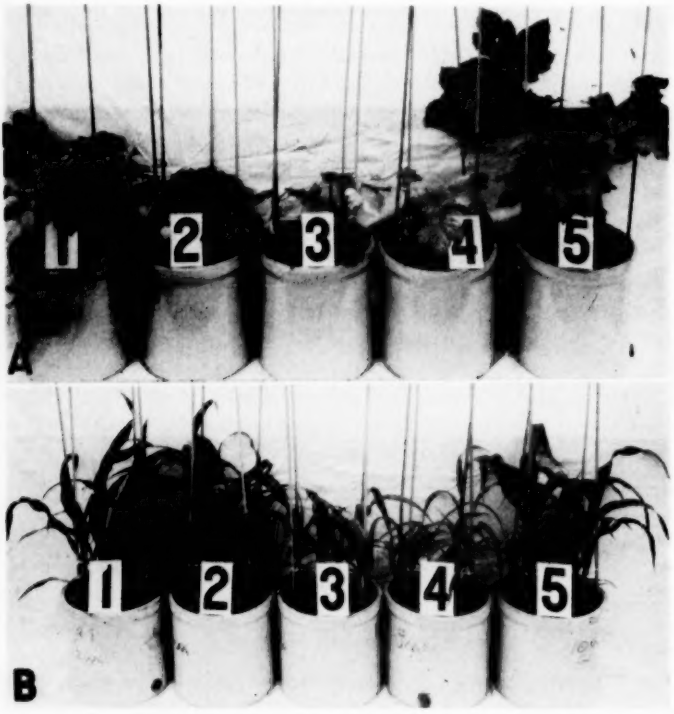

FIG. 1. A. Long Green okra on soils 1 to 5. Plants with limited copper supply on soils 3 and 4 and to a lesser extent soil 2 show interveinal chlorosis of lower leaves and retarded growth. B. Midland milo on soils 1 to 5. Plants with limited copper supply on soils 3 and 4 show a reddening of leaf tips, chlorosis and retarded growth.

Several duplicated experiments showed that the error between extractions of enzyme from the plant tissue was about 15%. The agreement of the extractions appeared to warrant comparisons between like material grown on the varied soil treatments. The plants were all grown, harvested, and the determinations made under like conditions.

Results

Wheat, barley, okra, milo, and cocklebur plants responded to copper but did not develop lime-induced chlorosis. Figures 1 A and B, and 2 A show

some of these plants and the nature of their response. Red kidney beans (fig. 2 B) and tobacco grew vegetatively well on the copper-deficient soil but did show some lime-induced chlorosis when grown on the calcareous soil. No chlorosis was evident in the spring harvested tobacco, but chlorosis did develop in the fall grown crop. None of the five soil treatments caused rye (fig. 3) to become chlorotic although the yield of plants grown on the calcareous soil was reduced.

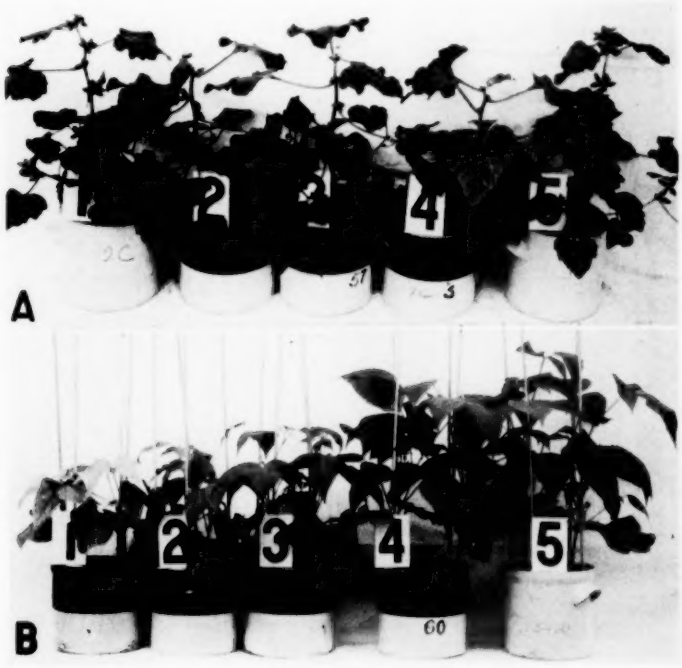

FIG. 2. A. Cocklebur grown on soils 1 to 5. Plants with limited copper supply on soils 3 and 4 failed to develop their burs and the leaves show necrotic areas. B. Red kidney beans grown on soils 1 to 5. Plants grown on calcareous soil 1 are chlorotic and retarded in growth. Plants grown with limited copper supply on soils 3 and 4 did not respond to copper.

Tables I and II give the yields and comparative enzymatic data for the above plants. The ascorbic acid oxidase activity was generally in agreement with the copper level of the growth medium regardless of the plant's showing a growth response to copper. Those plants which developed lime-induced chlorosis generally had a lower catalase activity than the nonchlorotic plants grown on the other treatments. Yield and enzymatic activity of tobacco appeared to be influenced by the season in which grown (fall or spring).

The visual deficiencies and enzymatic activities were not the same for all plants which responded to copper. Vegetatively, cocklebur showed very

TABLE I

YIELD AND RELATIVE ENZYME ACTIVITY OF WHEAT, BARLEY, MILO, OKRA, AND COCKLEBUR GROWN ON SOILS OF DIFFERENT IRON- AND COPPER-SUPPLYING CAPACITIES. ALL OF THESE PLANTS RESPONDED TO COPPER.

Crop (spring 1952)	Age	Soils									
		1 Calcareous		2 Two thirds calcareous one third organic		3 One third calcareous two thirds organic		4 Organic		5 Organic plus Copper	
	days										
						Catalase					
Thatcher sp. wheat	42	427		393		310*		241*		458	
Hannchen barley	44	181		228		199		193		272	
Hannchen barley	51	189		317		223*		169*		212	
Midland milo	....	61		68		70*		104*		112	
Long Green okra	48	203		202*		198*		192*		186	
Cocklebur	55	100		190		180*		180*		130	
						Peroxidase					
Milo	....	32		33		23*		36*		46	
Okra	48	74		68*		81*		57*		79	
Cocklebur	55	58		47		58*		49*		71	
						Ascorbic acid oxidase					
		Leaves	Nodes	Leaves	Nodes	Leaves	Nodes	Leaves	Nodes	Leaves	Nodes
Spring wheat	42	23	163	15	49	7*	32*	5*	15*	24	118
Barley	44	9	74	8	31	−2	33	−4	29	9	66
Barley	51	....	99	....	87	....	50*	....	1*	....	92
		Leaves		Leaves		Leaves		Leaves		Leaves	
Milo	....	15		34**		46**		29**		98	
Okra	48	97		69*		18*		15*		228	
Cocklebur	55	108		108		59*		45*		117	
						Average yield/plant† (Green wt.)					
Spring wheat	42	23		15		12*		20*		33	
Barley	44	29		25		26		24		43	
	51	37		32		24*		21*		47	
Milo	....	22		19		13*		13*		41	
Okra	48	31		8*		5*		8*		96	
Cocklebur											
Stem	55	26		39		44		43		37	
Leaves	55	39		47		55		62		49	
Burs	55	36		21		6*		5*		60	
Total weight		101		107		105		110		146	

*Severe copper deficiency symptoms.
**Endogenous activity very high.
†Average yield/10 plants for wheat and barley.

TABLE II

YIELD AND RELATIVE ENZYME ACTIVITY OF RYE, RED KIDNEY BEANS, TOMATOES, AND TOBACCO GROWN ON SOILS OF DIFFERENT IRON- AND COPPER-SUPPLYING CAPACITIES.

Crop	Date	Age	Soils				
			1 Calcareous	2 Two thirds calcareous one third organic	3 One third calcareous two thirds organic	4 Organic	5 Organic plus Copper
		days					
					Catalase		
Rye	March 26, 1952	46	350	365	343	261	300
Red kidney beans	March 12, 1952	33	153*	422	451	560	401
Tomatoes	May 6, 1952	62	70*	121	150	128	76
Tobacco	December 31, 1951	59**	188*	412	485	550	267
Tobacco	May 6, 1952	56**	192	208	212	190	103
					Peroxidase		
Rye	March 26, 1952	46	81	64	43	39	76
Red kidney beans	March 12, 1952	33	....	....	....	....	....
Tomatoes	May 6, 1952	62	14*	22	17	16	20
Tobacco	December 31, 1952	59**	33*	45	58	50	59
Tobacco	May 6, 1952	56**	38	56	43	43	37
					Ascorbic acid oxidase		
			Leaves Nodes	Leaves Nodes	Leaves Nodes	Leaves Nodes	Leaves Nodes
Rye	March 26, 1952	46	16 81	22 64	22 43	19 39	20 76
			Leaves	Leaves	Leaves	Leaves	Leaves
Red kidney beans	March 12, 1952	33	232*	160	91	9	336
Tomatoes	May 6, 1952	62	178*	125	98	57	123
Tobacco	December 31, 1951	59**	321*	313	79	6	303
Tobacco	May 6, 1952	56**	143	62	24	27	213
					Average yield/plant† (Green wt.)		
Rye	March 26, 1952	46	18	19	21	31	31
Red kidney beans	March 12, 1952	33	25*	30	34	69	69
Tomatoes	May 6, 1952	62	74*	105	120	156	230
Tobacco.	December 31, 1951	59**	350*	547	607	521	528
Tobacco	May 6, 1952	56**	746	754	855	851	885

*Lime-induced chlorosis.
**Days after transplanting.
†Average yield/10 plants for rye.

FIG. 3. Ottawa Select spring rye grown on soils 1 to 5. Rye did not develop deficiency symptoms on any of the treatments.

little response to copper, but as the plant became reproductive the burs failed to develop where copper was limited. Cocklebur thus differed from wheat, barley, okra, and milo which were all markedly reduced in growth. A very high endogenous activity was observed in determining ascorbic acid oxidase activity in copper-deficient milo plants. A poor correlation (table I) between visual deficiency symptoms and ascorbic acid oxidase activity was obtained for milo grown on soil treatments 1, 2, and 3. Treatments 4 and 5

TABLE III

PLANTS GROWN ON A CALCAREOUS AND ORGANIC SOIL, AND THE OCCURRENCE OF LIME-INDUCED CHLOROSIS OR COPPER-DEFICIENCY SYMPTOMS, RESPECTIVELY.

Plants	Calcareous soil (Lime-induced chlorosis)	Organic soil (Copper-deficiency symptoms)
Thatcher wheat	0	+
Hannchen barley	0	+
Long Green okra	0	+
Midland milo	0	+
Cocklebur	0	+
Red kidney beans	+	0
White lupine	+	0
Blue lupine	+	0
Yellow lupine	+	0
PI-54619-5-1 soybean	+	0
Connecticut broadleaf tobacco	+	0
Marglobe tomatoes	+	++
Minn Hybrid no. 800 corn	+	++
Hawkeye soybean	0	0
Ottowa Select spring rye	0	0

+ Visual deficiency symptoms other than retarded growth.
0 No visual deficiency symptoms.
++ Partial deficiency symptoms.

gave a good correlation. Milo appears to be limited in growth through a copper system, but not necessarily in the same manner as wheat and barley.

Discussion

The plants grown in this and a previous study (**1**) are grouped, table III, according to their response to the two types of nutrition investigated. From this grouping of plants it would appear that the plant's capacity to adjust to a particular environment might largely depend upon the plant's metabolic requirements. Wheat, barley, okra, milo, and cocklebur plants developed severe copper-deficiency symptoms, but none of these plants became chlorotic when grown on the calcareous soil. In contrast to the above plants, three lupines, soybeans, red kidney beans, tobacco, and rye did not respond to copper. This does not preclude some response to copper which might have been observed if these plants had been allowed to complete their reproductive development. The latter plants, except rye and soybeans, developed lime-induced chlorosis and appeared to be much more dependent upon available iron than copper.

Comparative enzymatic activities of ascorbic acid oxidase, catalase, and peroxidase, determined in this investigation, agreed with data from the previous study (**1**). The catalase activity was lower in lime-induced chlorotic than in normal green plants and there was no significant change in the peroxidase activity. If catalase activity is accepted as a possible index of active iron available in plant metabolism (**2**) lime-induced chlorosis appeared to be a true iron deficiency. Also, in view of the data available (**3, 5, 6**) which indicate that the iron concentration in chlorotic plants is about the same as that found in non-chlorotic plants, it seems probable that iron must be inactivated in some lime-induced chlorotic plants.

The cause of this inactivation or nonutilization of the iron present in the plant is yet to be determined. It seems possible that a different or altered metabolism in the plant may be a factor in determining the degree of susceptibility of plants to lime-induced chlorosis, since copper-requiring systems of metabolism seemed to predominate in most plants not susceptible to the chlorosis. The terminal oxidase in wheat and barley, two plants not susceptible to lime-induced chlorosis, has been determined by Waygood (**7**) and James and Cragg (**4**), respectively, to be ascorbic acid oxidase which might be a factor to enable these plants to utilize iron and grow on the naturally calcareous soil on which active iron becomes limiting in the chlorosis-susceptible plants. Iron-requiring metabolic systems appeared to predominate in plants susceptible to the lime-induced chlorosis and these plants were not copper responsive.

Plants grown on the calcareous soil seemed to have sufficient available copper as indicated by the relatively high ascorbic acid oxidase activity found in most of these plants. This available copper supply, in contrast to a low catalase activity or nonutilizable iron supply, may be an accentuating factor toward the development of lime-induced chlorosis. The average copper concentration in the leaves of 10 of the plants listed in table III was

6 p.p.m. in plants grown on the calcareous soil, 3 p.p.m. in plants grown on the organic soil, and 11 p.p.m. in plants grown on the organic soil to which copper sulphate was added (unpublished). Ascorbic acid oxidase activity appeared to be a good index to the available copper in most plants whether the plants did or did not show visual copper-deficiency symptoms. An increased activity of ascorbic acid oxidase with the addition of copper sulphate to the organic soil was observed in all of the plants studied.

Summary

Wheat, barley, Midland milo, okra, and cocklebur plants responded to copper when grown on the organic soil, but did not develop chlorosis on the calcareous soil. Red kidney beans and tobacco grew well on the copper-deficient organic soil, but did develop chlorosis on the calcareous soil. Tomatoes grown on the calcareous soil became chlorotic and also showed a small response to copper. Rye grew well on all of the soil treatments.

The catalase activity was lower in lime-induced chlorotic plants than in normal green plants but there was no significant change in the peroxidase activity. Ascorbic acid oxidase in the chlorotic plants was about the same as that in the normal plants.

Ascorbic acid oxidase activity appeared to be a good index of the available copper supply, whether the plant did or did not show visual copper-deficiency symptoms.

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RELATIONSHIP OF SEASONAL TRENDS IN CARBOHYDRATE AND NITROGEN LEVELS AND EFFECTS OF GIRDLING AND SPRAYING WITH SUCROSE AND UREA TO THE NUTRITIONAL INTERPRETATION OF BOLL SHEDDING IN COTTON

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The shedding of young cotton bolls has for many years been widely attributed to nutritional relations; insufficient carbohydrate and nitrogen supplies have most often been thought of in this connection. This view of the cause of boll shedding is possibly quite old, but the first reference with which the writers are familiar is that of MASON (**20**) in 1922 in which he said that both the cessation of growth of the main axis and the augmented susceptibility to shedding could be attributed to a correlation factor which tended to deflect the supply of elaborated food from the apical part of the plant to the fruit developing on the basal fruiting branches. In 1934 MASON and PHILLIS (**21**) wrote that the bolls drain the vegetative plant of its food materials, the leaves yellow and fall off as nitrogen is drained away, and as the strain increases the flower-buds and bolls themselves are starved and shed. In 1931, one of the present writers (**2**) stated that the nutritional dominance of bolls over the vegetative growth of the cotton plant had frequently been observed to inhibit boll setting and terminal bud and branch development. Eaton, in a section of a Field Station report by KING and LOOMIS (**17**) stated that a very close relation exists between the rate at which organic products are made available by photosynthesis and the number of bolls that the cotton plant retains. None of the foregoing conclusions was supported by original analytical data but it was known from earlier work that the nitrogen content of vegetative parts of the cotton plant declines with the advance of the summer. MASON (**20**) had found that the removal of all leaves caused a prompt shedding of nearly all young bolls carried by Sea Island cotton plants, and EATON (**2**) had found that when previously set bolls were removed from Upland plants these were replaced within a matter of 10 days with new bolls. HAWKINS *et al.* (**15**) measured the trends in carbohydrate levels in cotton plants throughout a summer and concluded in 1933 that the percentage of shedding is regulated by the amount of plant food available for the development of the young bolls. The conclusions drawn by WADLEIGH (**23**) in 1944 were that when developing ovules in cotton bolls already set have sufficiently depleted nitrogenous or carbohydrate reserves to a level of inadequacy for further ovule development, the additional young bolls abscise because of ovule abortion; after

a plant had set a number of bolls sufficient to deplete its nitrogen reserves, all subsequently formed young bolls abscised and, very soon thereafter, young squares abscised and the terminal buds of the fruiting branches aborted; there was little relation between percentage of abscission and treatment.

The present paper raises a question on the adequacy of the nutritional interpretation. Chemical and shedding data are presented on the effects of girdling and of spraying cotton plants with solutions of sucrose and urea and of combinations of these treatments and, also, on the seasonal march of carbohydrate and nitrogen levels in the cotton plant and its bolls through the fruiting period. The thought that spraying cotton plants with sucrose might supply a new approach to this old problem was suggested by remarks of F. W. Went in June 1947 when he reported outstanding growth responses with tomatoes sprayed with sucrose solutions. WENT and CARTER (**25**) have reviewed the literature on the uptake of sugars from solutions. In one of their experiments in a dark room, unsprayed tomato plants grew 15.8 mm. in six days; plants sprayed on the upper sides of the leaves with 10% sucrose grew 27.5 mm. and those sprayed on the underside grew 37.0 mm. Another of their experiments showed that the entry of sucrose was not dependent upon open stomata; the stomata in the tomato, as in cotton, are more numerous in the lower than in the upper epidermis. The authors pointed out that the upper cuticle is thicker than the lower and, also, that the concentration of the sucrose spray should make little difference since the concentration always increases as evaporation progresses.

Urea sprays have been tested for a longer period than sucrose sprays. In general (**6, 11, 12, 16, 20**) plants on soils that respond to soil applications of nitrogen also respond to urea sprays and sometimes more promptly and with certain advantages. EMMERT and KLINKER (**6**) have reported that the tendency for urea sprays to cause leaf burning is avoided when sufficient sucrose is added to the solution. Because of the current interest in both sucrose and urea sprays, the results of the experiments involving these are given more attention in this paper than they would otherwise have received.

Methods

The sucrose and urea spray experiment of 1947 at Shafter, California, was with Missdel × Acala $F_8$10-13 plants in six-foot row segments for each plot in five randomized blocks. There was an average of 6.2 plants per plot, extra plants were treated at the end of each plot to provide sample material. Previously set bolls (an average of one per plant) were removed on July 3. All subsequent flowers and shed bolls were counted daily. The plants were sprayed six days per week, usually in the late afternoon, starting on July 3 and continuing until August 26. Samples for analyses consisted of four leaf blades from the middle third of the main stem of each of 12 plants, the petioles of these leaves, the middle third of the main stems of the same 12 plants and 13-day-old bolls (10 to 14 per sample). These samples were collected

on July 16, July 31, and August 14. On the collection dates, half of the samples were taken in the late morning and half in the early afternoon. Plant heights were measured weekly. Before weighing and drying, the samples were agitated in two changes of water to remove surface materials. In spraying, a spreader was used and the upper surfaces of all leaves were thoroughly wetted. The four treatments were 20% sucrose spray, 1% nitrogen (Nugreen) spray, 20% sucrose plus 1% nitrogen, and water spray only. The plots were irrigated weekly.

The 1948 experiment was also with a Missdel × Acala strain and as in 1947 the plots were irrigated weekly. The treatments were started on August 14 and continued for 11 days. Plants were treated in four ways: (a) sprayed each second day on their upper leaf surfaces with 2% nitrogen (six times), (b) girdled above a large fruiting branch, (c) both girdled and sprayed as above, and (d) untreated. There were 10 consecutive plants for each treatment in each of four blocks with extra treated plants in each plot for samples which were collected on August 21. By August 21, few of the girdles had been bridged by new tissue. Duplicate samples from eight plants from each treatment were collected, one sample being from the upper two blocks and one from the lower two. The root samples were composed of the tap and larger secondary roots as lifted with a shovel. The leaf and stem samples were from the middle third of the main stalks. Each day's flowers (August 14 to 24 inclusive) were counted and marked with a different colored tag. On August 29 the tags from the healthy bolls remaining on the plants were collected for the boll-shedding record.

The carbohydrate and nitrogen analyses were made by methods previously described (**5, 7**). The organic acids in the leaves were determined by methods described by PUCHER *et al.* cited elsewhere (**8**).

Results

EFFECTS OF SUCROSE AND UREA SPRAYS IN 1947

The carbohydrate and nitrogen concentrations found in the plants of the spray experiment in 1947 are reported in table I as the averages of the three collections. The effects of the treatments on plant growth, flowering and boll retention are shown in table III. Starting during the second week of spraying, there was evidence of marginal burning of the leaf blades sprayed with urea alone. This burning progressed and by late August the plants sprayed with urea alone had about half the leaf area of the other treatments. When the same amount of urea was used with 20% sucrose there was no burning; the latter plants, like the sucrose-sprayed plants, had the same appearance as the control (water spray) plants. Table I shows that far less nitrogen was accumulated in the leaves when the urea was combined with sucrose than when the urea was used alone. This depression in urea absorption would account for the absence of burning.

When the experiment was started, a gummy coating of sucrose (both sucrose alone and sucrose plus urea) remained on the surface of the leaves

from one day to the next. But early in the second week, it became difficult to distinguish the leaves that had been sprayed the evening before with sucrose from those that were sprayed only with water. The samples were collected in moist cotton bags and kept in the shade until they could be taken to the laboratory for washing and weighing. On one of the sample dates, the plants had been sprayed in the early morning; the leaves when collected were coated with sucrose. When taken from the bags for washing those sprayed with sucrose were found to be very wet. The solution on the surface of these leaves (not found on the leaves treated with urea or water)

TABLE I

CARBOHYDRATE AND NITROGEN LEVELS IN VARIOUS PARTS OF COTTON PLANTS SPRAYED WITH WATER, SUCROSE AND UREA. PERCENTAGES ON FRESH WEIGHTS. MEANS OF COLLECTIONS OF JULY 16, JULY 31, AND AUGUST 14.

Plant part	Spray treatment	Moisture	Carbohydrates					Nitrogen	
			Hexose	Sucrose	Starch	Sum	Hemicellulose	Soluble	Insoluble
Leaf blades	Water only	80.7	0.05	0.13	0.28	0.46	0.76	0.16	0.54
	Sucrose	80.7	.07	.15	.31	.53	.83	.14	.58
	Urea	79.6	.01	.02	.20	.23	.83	.32	.73
	Sucrose and urea	80.1	.07	.09	.22	.39	.84	.21	.60
	L.S.D. 5%	.09	.08	.10	.19	.20	.06	.08	.23
Petioles	Water only	82.9	.09	.15	.26	.50	....	.20	.11
	Sucrose	83.8	.08	.24	.26	.58	....	.20	.08
	Urea	83.4	.06	.12	.17	.36	....	.24	.08
	Sucrose and urea	83.9	.10	.10	.24	.44	....	.17	.08
	L.S.D. 5%	1.0	.03	.14	.06	.18	....	.03	.05
Stems	Water only	75.4	.22	.56	.56	1.34	....	.10	.12
	Sucrose	77.9	.14	.45	.47	1.04	....	.09	.12
	Urea	77.3	.11	.38	.43	.93	....	.12	.13
	Sucrose and urea	76.7	.15	.44	.48	1.15	....	.11	.12
	L.S.D. 5%	3.9	.08	.19	.15	.35	....	.02	.05
13-Day Bolls	Water only	87.1	1.24	.04	.88	2.16	....	.12	.18
	Sucrose	87.6	1.26	.02	.79	2.08	....	.11	.18
	Urea	86.7	1.06	.04	.90	1.97	....	.15	.21
	Sucrose and urea	87.1	1.17	.02	1.01	2.21	....	.13	.20
	L.S.D. 5%	.85	.52	.04	.19	.58	....	.026	.026

was apparently the result of leaf water withdrawn by osmosis from inside the leaves.

The dew-point was reached during about half of the nights of the experiment and it was approached on the remaining nights. With this high nightly humidity it seems evident that there was enough moisture withdrawal by osmosis from leaves to let the sucrose solution drip to the ground. This runoff caused by osmosis apparently did not occur until after the surface cuticle had been altered during the first week of spraying. The analytical data of table I show little evidence that sucrose was absorbed. The observations are pertinent, nevertheless, that between about 4 P.M. and 4 A.M.

there was an opportunity for epidermal uptake of sucrose and that after the first week the epidermis was permeable, at least to water. Computations made at several stages of the experiment showed that enough sucrose was being applied to have supported double the number of bolls being developed by the plants.

Sucrose when used as a spray alone (table I) gave slight nonsignificant increases in the carbohydrates of the leaves and petioles. These increases, however, were balanced by non-significant losses in the stems and bolls 13 days old. There was no significant effect of sucrose spray on either of the soluble or insoluble nitrogen fractions in any of the tissues.

Urea used alone reduced the sugar and starch in leaves, petioles and stems and these reductions were for the most part significant. There was a small loss of carbohydrate in the bolls as well. With only one exception (insoluble nitrogen in petioles), the urea spray increased both soluble and insoluble nitrogen in all tissues; these nitrogen gains were usually significant. Some of the carbohydrate reduction associated with the urea spray is reflected in gains in the carbon content of the nitrogen compounds. Using for purposes of comparison, the factor 6.25 to convert the total nitrogen to protein, there was in the leaves a gain of 2.18% in terms of protein equivalent against the reduction of 0.23% found for carbohydrate. In the stems the protein gain failed to compensate for the carbohydrate loss. In the bolls, the loss of carbohydrate was 0.19% and the protein gain was 0.38%. The spray of sucrose and urea resulted in slight non-significant losses in total carbohydrate (a small gain in the bolls) and slight gains (petioles excepted) in total nitrogen. All of the sprays caused significant increases in hemicellulose concentrations in the leaves.

In general, it would seem that the effect of the sucrose spray and the sucrose and urea spray on the carbohydrate concentrations in the plants were too small to merit consideration. Some interest can be attached to the increased nitrogen but lowered carbohydrate concentrations in all tissues resulting from the use of the urea spray alone. There are yet to be considered some analyses (table II) of the concentrations of various organic acids in the leaves. It has been recurrently suggested that the organic acids may be involved in the initiation of reproductive processes.

All of the sprays reduced the concentrations of citric and malic acids (table II). The effects of the urea spray in reducing malic acid and of the sucrose spray in increasing the unidentified acids were especially marked. The reduction in malic acid by the urea spray was not compensated by gains in the other acids and the loss shows up significantly in the total organic acids. The low total organic acids found in the leaves sprayed with urea are associated most logically with reduced concentrations of bases which have been found in other plants as well as in cotton (**8**) when fertilized with ammonium salts. Any association between changes in the organic acids and the retention of bolls, if such existed, might be the significant loss in citric acid and gain in the unidentified acids that resulted from the sucrose spray.

TABLE II

ORGANIC ACIDS OF LEAVES. DATA EXPRESSED AS MILLIGRAM EQUIVALENTS PER 100 GRAMS DRY WEIGHT.

Treatment	Citric	Malic	Oxalic	Unidentified	Total
Water only	77.2	99.5	11.7	24.1	202.4
Sucrose	58.6	87.0	12.3	37.3	195.3
Urea	62.0	70.9	11.7	26.3	170.1
Sucrose and urea	65.8	89.3	11.5	28.9	196.0
L.S.D. 5%	16.7	11.6	0.6	4.7	28.4

As shown in table III, there was an apparent disadvantage from all the sucrose and urea spray treatments on plant height, but these differences lacked statistical significance. There were no significant effects of the various sprays on the number of flowers produced. Each of the three spray treatments reduced the number of bolls retained; in each case, these reductions were significant. The trends of the four treatments in flower production and boll shedding were noted to follow similar courses, *i.e.*, the three sets of sprayed plants remained like the controls in flowering rates but gradually fell below in boll retention. It seems evident that if any of the sucrose sprayed on the leaves was taken up by the plants it was offset by reduced photosynthesis.

This experiment like the one that followed in 1948 was conducted under conditions of high nitrate supply. The irrigation water at Shafter, California, adds 25 pounds of nitrate nitrogen per acre foot of application. About four acre feet of water, or 100 pounds of nitrogen, are applied annually and cotton at the Shafter Station has shown no response to additional applications.

Effects of girdling and urea sprays in 1948

The girdling and spraying with urea in this experiment were done at a time when the cotton plants were shedding over half of their bolls. The girdle was cut above one of the lower fruiting branches that had four or five leaves; special care was taken to avoid injuring the xylem as much as possible. A pair of razor blades were set 5 mm. apart in a block of wood

TABLE III

GAIN IN PLANT HEIGHT (JULY 4 TO AUGUST 25), AND NUMBER PER PLOT (ABOUT SIX PLANTS) OF FLOWERS (JULY 3 TO AUGUST 21) AND OF BOLLS RETAINED (JULY 3 TO AUGUST 27).

Spray treatment	Height	Flowers	Bolls retained
	cm.		
Water only	51	188	80
Sucrose	47	189	57
Urea	43	203	69
Sucrose and urea	41	204	65
L.S.D. 5%	11	34	11

with the edges protruding only far enough to cut just through the bark of most of the stems. As soon as the strip of bark was removed, the wound was wrapped with scotch tape. The girdles were cut, the first urea spray applied, and the tagging of flowers was started on the same day. The samples for chemical analyses were collected a week later. On the eleventh day, none of the plants showed any appearance of having been injured by the girdling and there was at that time only minor marginal leaf burning from the spray of 2% nitrogen applied each second day (six times in all).

The urea spray (both with and without girdling) more than tripled the soluble nitrogen of the leaves and almost doubled that in the stems (table IV). There was a substantial increase in protein (insoluble) nitrogen in the leaves but not in the stems. Urea spray, relative to the control plants, and

TABLE IV

EFFECT OF UREA SPRAY AND GIRDLING ON CARBOHYDRATE, NITROGEN, PHOSPHORUS, AND POTASSIUM CONCENTRATIONS. THE SAMPLES WERE COLLECTED ONE WEEK AFTER STARTING THE TREATMENTS. DATA ARE EXPRESSED AS PERCENTAGES ON FRESH WEIGHT.

Part of plant	Treatment	Moisture	Carbohydrates				Nitrogen		Phosphorus	Potassium
			Hexose	Sucrose	Starch	Sum	Soluble	Insoluble		
Leaves	Control	77.6	0.12	0.19	0.40	0.71	0.14	0.60	0.056	0.047
	Urea	75.4	.02	.04	.36	.42	.51	.72	.060	.044
	Girdle	77.6	.12	.20	.58	.90	.15	.54	.048	.038
	Urea and girdle	74.5	.06	.06	.49	.61	.48	.69	.052	.042
Stems	Control	74.0	.16	.70	.31	1.17	.06	.11	.032	.48
	Urea	72.6	.08	.44	.28	.80	.11	.11	.039	.54
	Girdle	71.0	.28	1.46	.77	2.51	.05	.11	.020	.41
	Urea and girdle	70.0	.21	.80	.96	1.97	.12	.13	.024	.41
Roots	Control	71.2	.20	1.26	1.08	2.54	.04	.10	.046	.38
	Urea	71.2	.14	.98	1.10	2.22	.06	.10	.050	.40
	Girdle	71.9	.14	.74	.60	1.48	.06	.09	.038	.34
	Urea and girdle	71.2	.14	.64	.66	1.44	.05	.09	.042	35

the urea and girdling treatment, relative to the girdled plants, caused 25 to 40% reductions in the sums of sugars and starch in the leaves and stems. Girdling alone increased the concentrations of sugars plus starch by 27% in the leaves and by 100% in the stems. In the large roots there was a 60% reduction in carbohydrate. Treatment with urea and girdling, relative to the control plants, reduced the carbohydrate in the leaves a little, but increased that in the stems from 1.17 to 1.97%; this increase was associated with an increase from 0.17 to 0.25% in total nitrogen. During the period of the observations, flowering rates (table V) were not influenced by any of the treatments. Relative to the control plants the urea spray reduced the number of bolls retained to one half. Girdling reduced the number of bolls retained to one third. Urea and girdling together reduced the number to one fifth. Relative to the control plants all of these reductions were statistically

significant. Neither the large increase in nitrogen from urea treatment and the carbohydrate gain from girdling, nor the increases in both from urea and girdling increased boll retention *per se;* instead there were large decreases.

Seasonal trends in carbohydrates levels

As a further basis for evaluating possible effects of carbohydrate and nitrogen levels on boll shedding, there have been assembled in table VI, from other experiments, results showing the seasonal march of carbohydrate concentrations in the middle third of the main stems of five varieties (12 plants per sample) at College Station, Texas, in 1945 and in three varieties at Shafter, California, in 1947. At Shafter, data were obtained on nitrogen, as well as carbohydrate concentrations, in leaves (four leaf blades from the middle third of each of the 12 stems) and in 13-day-old bolls (12 bolls usually from the above plants). In both years, the plants were sampled about July 15, August 1, and August 15. At College Station the plants were sampled from randomized plantings in mid-afternoon. At Shafter, half of

TABLE V

FLOWERS PER 10 PLANTS DURING PERIOD OF SPRAYING, AUGUST 14 TO 24 INCLUSIVE, AND NUMBER OF HEALTHY BOLLS FROM THESE THAT REMAINED ON PLANTS ON AUGUST 29.

Treatment	Flowers	Bolls retained
Control	78	30
Urea	69	15
Girdle	71	9
Urea and girdle	77	6
L.S.D. 5%	....	7

the samples were collected in mid-morning and half in mid-afternoon. In the instance of the Stoneville and Acala varieties, the sampling was divided between two consecutive days reversing the order of collection in the two morning and two afternoon samplings. During both years, the plants had started to flower in early July and were fruiting rapidly and retaining nearly all of their bolls on July 15. By August 1, there was a little shedding but most of the bolls were being retained. By August 15 over half of the bolls were being shed. All plants were grown in plots irrigated weekly.

The middle third of the main stem provides a creditable index tissue for carbohydrate levels in the cotton plant. As shown in table VI, carbohydrates accumulate in the stems in concentrations three times those of the leaves. Carbohydrates being moved downward to the roots and much of those interchanged between the fruiting branches pass through this portion of the plant. Bolls 13 days from flowering were used because at this age they are well beyond the stage that they might be failing to develop prior to shedding; also they are five to eight days ahead of carbohydrate utilization in oil synthesis in the seed and in cellulose deposition in the secondary thickening of the walls of the lint cells.

The large decrease in the carbohydrates of the Missdel × Acala strain in late July (table VI) is perhaps of some general interest in that the strain had been selected by G. J. Harrison for its high resistance to Verticillium wilt; but Stoneville and Acala plants with bolls removed to increase their carbohydrates also appeared to have some extra resistance. In another paper (**3**) in which some of these data were used, attention has been called

TABLE VI

CARBOHYDRATE AND NITROGEN LEVELS THROUGH THE PERIOD OF BOLL SETTING. THE FIRST SAMPLES WERE TAKEN ABOUT JULY 15 (EARLY FRUITING), AUGUST 1 (HEAVY BOLL SETTING), AND AUGUST 15 (HEAVY BOLL SHEDDING). DATA EXPRESSED AS PERCENTAGES ON FRESH WEIGHT.

Location, tissue, and variety	Total sugars			Sum of sugars and starch			Total nitrogen		
Sampling:	1	2	3	1	2	3	1	2	3
College Station, 1945									
Stems, middle									
Arkansas Rowden B 4	2.05	1.83	1.44	2.52	2.97	3.77	....	....	....
D & Pl 14	2.03	1.97	1.95	2.97	3.03	3.25	....	....	....
Hi-Bred	1.10	2.04	2.09	1.83	4.02	3.97	....	....	....
Stoneville 2 B	2.13	2.35	2.30	2.83	3.73	3.69	....	....	....
Oklahoma Triumph 92	2.03	2.36	1.58	2.75	4.02	3.80	....	....	....
Shafter, 1947									
Stoneville 2 B									
Leaves	.58	.49	.46	1.09	.87	.78	0.84	0.75	0.78
Stems, middle	1.03	1.51	1.36	1.38	2.25	2.31	.25	.24	.20
Bolls, 13 days	1.85	1.51	1.31	3.33	2.73	2.46	.28	.26	.25
Acala p 18									
Leaves	.36	.51	.23	.99	.97	.69	.86	.77	.72
Stems, middle	1.46	1.37	.94	1.79	1.96	1.63	.25	.18	.13
Bolls, 13 days	2.04	1.61	1.02	3.50	2.69	1.79	.29	.18	.13
Missdel × Acala									
Leaves	.25	.13	.15	.74	.23	.42	.81	.61	.66
Stems, middle	1.22	.50	.41	2.10	1.06	.88	.29	.21	.18
Bolls, 13 days	1.49	.93	1.40	2.47	1.51	2.50	.32	.33	.19
Means:									
College Station									
Stems	1.87	2.11	1.87	2.58	3.55	3.69	....	....	....
Shafter									
Leaves	.40	.38	.28	.94	.69	.63	.84	.71	.72
Stems	1.25	1.13	.90	1.76	1.76	1.60	.26	.21	.17
Bolls	1.79	1.35	1.24	3.10	2.31	2.25	.30	.26	.19

to significant differences in carbohydrates between the five varieties at College Station and, also, to the fact that the Stoneville at Shafter had only 60% as much sugar plus starch as at College Station.

Directing attention to the levels of carbohydrates in the stems of the Stoneville and Acala varieties at Shafter, there was a substantial gain in the sugars of Stoneville (1.03 to 1.36%) between mid-July and mid-August and during the same period there was a somewhat larger loss (1.46 to 0.94%) in Acala. Between these dates starch increased in Stoneville from 0.35 to

TABLE VII

CARBOHYDRATE AND NITROGEN CONCENTRATIONS IN THE MIDDLE THIRD OF COTTON STEMS AND IN BOLLS 13 DAYS OLD IN SAMPLES FROM FIRST, SECOND AND THIRD PLANTINGS. DATA EXPRESSED AS PERCENTAGES ON FRESH WEIGHT.

Variety and planting	Sucrose			Total Sugars			Sum sugars and starch			Total nitrogen		
Sample dates:	July 14–15	July 29–30	August 11–12	July 12–15	July 29–30	August 11–12	July 14–15	July 29–30	August 11–12	July 14–15	July 29–30	August 11–12
Wet												
Stems												
Stoneville 2 B												
First planting	0.89	1.25	1.02	1.03	1.51	1.36	1.38	2.25	2.31	0.25	0.24	0.20
Second planting		.81	.92		1.27	1.29		2.11	2.14		.22	.21
Third planting			.73			1.35			2.28			.19
Acala p18												
First planting	.24	1.06	.69	1.46	1.37	.94	1.79	1.96	1.63	.25	.18	.13
Second planting		.76	.98		1.06	1.27		1.53	1.74		.18	.16
Third planting			.81			1.17			1.62			.18
Bolls												
Stoneville 2 B												
First planting	.08	.00	.07	1.85	1.51	1.31	3.33	2.73	2.46	.28	.26	.26
Second planting		.04	.01		1.10	1.41		2.51	2.74		.24	.27
Third planting			.05			1.32			2.95			.26
Acala p18												
First planting	.00	.00	.02	2.06	1.61	1.02	3.50	2.69	1.79	.29	.29	.13
Second planting		.04	.07		1.75	1.69		3.00	2.01		.26	.10
Third planting			.00			1.18			2.22			.30
Means—3 moistures												
Stems												
Stoneville 2B												
First planting	.66	.76	.79	1.20	1.06	1.18	2.07	2.13	1.86	.24	.18	.20
Second planting		.85	.89		1.37	1.31		2.27	2.44		.22	.20
Third planting			.88			1.37			2.11			.19
Acala p18												
First planting	.84	1.01	0.72	1.41	1.37	1.01	2.26	2.96	1.90	.26	.20	.19
Second planting		.84	1.15		1.28	1.47		1.94	2.31		.18	.17
Third planting			.78			1.13			1.59			.17
Bolls												
Stoneville 2B												
First planting	.03	.02	.05	2.02	2.42	1.46	4.17	3.12	2.70	.29	.26	.25
Second planting		.01	.12		1.42	1.79		2.71	3.32		.25	.26
Third planting			.03			1.26			2.57			.25
Acala p18												
First planting	.05	.02	.01	2.08	1.87	1.14	3.80	3.04	2.23	.30	.29	.23
Second Planting		.05	.05		1.31	1.44		2.50	2.36		.29	.28
Third planting			.00			1.09			2.27			.28

0.95% and in Acala from 0.33 to 0.69%. Both varieties, it will be recalled, were retaining most of their bolls in mid-July and shedding most of them in mid-August. At College Station, with a similar shedding performance, there was no mid-July to mid-August change in the five-variety sugar average but there was an average gain in starch from 0.71 to 1.82%. These data fail to provide a carbohydrate explanation for the boll shedding in August. The carbohydrate data for the bolls 13 days old are yet to be considered.

Nitrogen concentrations were found to follow a downward course in all tissues in California and, in keeping with earlier data, this was probably the case at College Station also. The possible significance of the declining nitrogen levels in connection with boll shedding should be considered with the fact that in the short experiment of 1948, the urea spray increased nitrogen and caused more rather than less shedding. Neither at College Station nor at Shafter were the cotton yields increased by nitrogen fertilization.

Step-by-step decreases occurred in the young bolls in the concentrations of both sugars and starch in going from mid-July to mid-August (table VI). In mid-July there is evidence of rather strong polar transport from the leaves to the stems and from the stems to the bolls. In both varieties the carbohydrate differentials between the stems and bolls had almost disappeared by mid-August. This failure of the 13-day-old bolls to maintain favorable sugar differentials relative to the stems might appear on the basis of the evidence of table VI to provide an explanation of the increased shedding of young bolls as the cotton plant becomes older. The data of table VII provide an opportunity for a further examination at this point.

In 1947, there were two plantings of both the Stoneville and Acala varieties that were made later than the one that supplied data for table VI. Each of the three plantings was grown at three moisture levels: wet, irrigated on each side of the rows each week; intermediate, irrigated on one side each week; and dry, irrigated on one side each second week.

The three plantings were three weeks apart. The second planting started flowering 15 days after the first and the third 15 days after the second. Three sets of samples (table VII) were collected from the first planting, two from the second planting and one from the third; all three plantings were sampled at the last date and the first two at the intermediate date. For the four-day periods preceding and including July 14, July 29, and August 11 the average maximum temperatures were 93, 95, and 95° F respectively, the minimum temperatures were 60, 62, and 65°, the relative humidities 34, 34, and 32%, and the day length 14.4, 14.1 and 13.7 hours. The respective mean values for the actual sampling days were maximum 96, 96, and 98, minimum 62, 63, and 64, and humidity 33, 35, and 32. As computed by T. H. MacDonald of the U. S. Weather Bureau, the mean clear-day horizontal-plane total radiation for the period June 30 to July 19 was 770 gram calories per sq. cm. per day and 700 gram calories for the period July 26 to August 14.

The carbohydrate and nitrogen data are presented both on the basis of the results in the wet plots and on the basis of the means of wet, intermedi-

ate, and dry treatments. Each of the three plantings of both varieties of cotton retained nearly all bolls during the first weeks of their flowering. By mid-August, the first plantings of both varieties were shedding most of their new bolls whereas the last plantings were retaining their bolls.

In the first planting, the concentration of total sugars in both the Stoneville and Acala bolls was higher in mid-July than at later dates and higher also than any levels reached by the later plantings. For the means of the three levels of moisture supply in mid-August, the concentrations of sugar and starch, and under the wet treatment the concentrations of sugars, were equally low in the stems and in the 13-day-old bolls of the first and last plantings. Also in the means, only minor differences were found in mid-August in the concentrations of nitrogen in the two plantings. This lack of difference between the young and old plants in the level of carbohydrate and nitrogen supply in the stems and in the operational levels within their 13-day-old bolls affords no insight as to why the older plants should have been shedding their bolls profusely at the same time the young plants were retaining their bolls.

The interpretation of the data for the bolls might have been aided had bolls during the first days of their development been analyzed; but during this early period there is no certain basis for distinguishing between bolls that are going to shed (these presumably would be low in carbohydrate) and the bolls that will be retained. The general observation is accordingly appropriate that whether or not carbohydrate levels within very young bolls are causally involved in shedding, antecedent differences are not shown in the stems of the shedding and non-shedding plants nor are differences reflected in the 13-day-old bolls of such plants.

Discussion

Some of the literature on the shedding of cotton bolls is pertinent to the present discussion. Similar percentages of boll shedding have been found with variations in manuring and fertilization. Ewing (**10**) obtained widely different growths and numbers of flowers on poor soil and on rich bottom soils, but the percentages of bolls that shed were much the same. In a less striking field experiment (**22**) similar percentages of shed bolls were found when the proportions and amounts of N, P, and K were varied.

Data on the carbohydrate and nitrogen accumulations within the plant in relation to NO_3 supply are available. Wadleigh (**23**) using four nitrate levels in sand cultures in a whitewashed greenhouse in the summer, pointed out that regardless of treatment 60 to 70% of all blossoms produced were shed and gave thought to the possible bearing of nitrogen and carbohydrate deficiencies on shedding. Calculations from Wadleigh's data have shown that for his four NO_3 levels there were respectively 3.6, 3.5, 2.8, and 3.5 bolls per 100 grams of fresh stems and leaves. Eaton and Rigler (**5**) also used four NO_3 levels in sand cultures—the highest one being four times as great as Wadleigh's highest. The experiment was repeated under muslin shades

in a greenhouse in the winter and fully exposed outdoors in the summer. The four NO_3 treatments gave an average of 12% of bolls shed under low light and of 40% under high light. At low light, the root bark contained an average of 1.16% of sugars plus starch and the plants had an average relative fruitfulness of 4.0 bolls per 100 grams of fresh stems and leaves; under high light the root bark contained 2.5% of sugars plus starch and the relative fruitfulness was 6.8 bolls per 100 grams of stems and leaves. Under low light there was a heavy shedding of floral buds and under high light there was very little. Both the high and low nitrate levels reduced sharply the sizes of the plants but at corresponding nitrate levels the stems and leaves weighed almost the same under low and high light. The light intensity thus altered relative fruitfulness but neither it nor the associated changes in carbohydrate influenced vegetative weight. In both of the experiments, the NO_3 treatments that resulted in larger plants also resulted in proportionately more bolls. In the interpretation of this experiment, it seemed pertinent to ask why, if carbohydrate supply limited boll setting under low light, the high-light plants did not reduce their carbohydrate supply down to the low-light level before these plants started to shed.

From the foregoing experiments, the conclusion seems to be supported that neither the carbohydrate or nitrogen levels in the plants influence relative fruitfulness very much, *i.e.*, the partition of growth between vegetative and reproductive activities. Within given light intensities, the partition of carbohydrate utilization remained quite uniform but between intensites there were marked differences. Neither carbohydrate nor nitrogen levels in the plants nor the level of NO_3 supply seem in the above experiments to have constituted critical factors in boll shedding but this evidently does not apply to the shedding of floral buds.

Something comparable to the foregoing was found (**11**) when the sulphate supply for cotton plants in greenhouse sand cultures was increased from a low level, that resulted in little or no sugars in the leaves and stems and very little growth, to a high level that gave large plants with normal sugars and many bolls. Over the SO_4 range there were successively 3.8, 4.0, 3.8, and 4.0 bolls per 100 grams of fresh stems and leaves.

The present experiments with urea and sucrose sprays, used alone and together, and with girdling and urea sprays, alone and together, were found to have had consistently adverse effect on boll retention. The sucrose spray failed to increase carbohydrate levels in the plant but the urea spray when used alone decreased carbohydrate and increased nitrogen. Girdling increased carbohydrate levels in the plant stems materially and when combined with urea spray increased both carbohydrate and nitrogen. The organic acids of the cotton leaves were altered by the sucrose and urea sprays but if these changes were related to boll shedding the effect was adverse, or at least masked by other effects of the treatments. Had the foregoing treatments produced neutral effects on boll retention, they could be more readily comprehended; the strongly adverse effects are difficult to understand.

During the course of the investigations, it has been felt that a great weakness in the nutritional theory of boll shedding, as regards carbohydrate and nitrogen levels in plants, rested in the fact, as illustrated in table VI, that the seasonal march in shedding rates is not correlated with the seasonal march in stem carbohydrates. The results in the short-period experiment of 1948 with urea spray, which greatly increased nitrogen in the tissues but caused only a trace of leaf injury, have tended to make it seem unlikely that the seasonal downward trend in nitrogen levels in the cotton plant were responsible for late season shedding. As nitrogen becomes deficient, both vegetative growth and flowering are slowed down together.

Examinations of very young bolls for carbohydrate and nitrogen contents during the period when they might or might not be going to shed is impracticable because changes prior to abscission may take place before the slowing of growth can be noted. The use of 13-day-old bolls for analysis, it was believed, might avoid the weakness attached to analyzing very young bolls and thereby provide a basis for judging whether important differences exist in the carbohydrate relations of the bolls themselves during the early season when nearly all bolls are being retained and the late season when nearly all are shedding.

When 13-day-old bolls from plants in their early, medium and late fruiting period were analyzed, step-by-step decreases in carbohydrate levels were found as the plants became older. The least carbohydrate was found in these bolls at the time when the plants were shedding most heavily. Early in the fruiting period, the bolls had much higher concentrations than the stems, but by mid-August the concentrations of the stems and bolls were nearly equal; this suggested that plant-age factor was involved in polar transport and the possibility that transport of carbohydrate to the bolls might be a critical factor in boll retention. An extension of the same experiment, however, included later plantings of the same two varieties of cotton and these data did not support this suggestion. These later plantings provided stems and bolls for collection from old plants that were shedding and from young plants that were not, all on the same days of sample collections in mid-August. The results of these measurements were rather surprising in that nearly the same concentrations of carbohydrate and nitrogen were found in mid-August in the bolls 13 days old from the old and from the young plants. Time or season of sampling rather than plant age, or shedding performance, seemed to determine the carbohydrate levels in the bolls. There was very little difference in maximum and minimum temperatures or humidity on and preceding the three sampling dates but there was a reduction in day length. If day length was the critical factor in the carbohydrate accumulation in the stems and bolls, the reason, or mode of action, remains obscure. American Upland cottons are day-length neutral. The photoperiodic cottons express their day-length reactions primarily in the retardation until short days of the development of their first fruiting branches. The first fruiting branches of the short-day cottons often shed many of the early floral buds.

Recognizing that the carbohydrate level found in a plant tissue is only the resultant between the diverse processes of photosynthesis, respiration, translocation, and conversions to proteins and other materials, the low level of carbohydrate found in the young bolls in mid-August might thus be considered as being the result of more rapid utilization. This explanation seemingly will not hold in the present instance; bolls set in mid-August require longer to mature than those set in mid-July and they are smaller when mature.

The early observation (**2**) that the removal of all bolls from cotton plants was followed promptly by stimulated vegetative growth and a rapid replacement of the bolls has seemed to support the nutritional interpretation of boll shedding. Since, as shown in the present paper, the period of heavy boll shedding in cotton is not accompanied by any diminution in the carbohydrate levels in the plant stems, it seems appropriate to give brief consideration to some of the points in boll shedding that would need to be reconciled in any alternate interpretation. Small floral buds die in place rather extensively when cotton plants are grown under low light intensities. This death of buds (as distinct from the usual boll shedding) has been attributed to the associated low levels of carbohydrates in the plants. Also, there is a special characteristic of boll shedding which is always of interest; namely, that it is only during the first week or so following anthesis that bolls customarily abscise. It is extremely rare to find large floral buds shedding prior to anthesis. The uniformity found in the relative fruitfulness measurements under given environmental conditions, but over wide ranges of nutrient supply, leads to the idea of a balance between the number of leaves on cotton plants and the number of bolls that are set or, as a possible corollary, a balance between some mobile anti-auxin type of material developed in growing cotton bolls and the auxin produced in cotton leaves. A number of investigators have studied the effects of organic chemicals on boll abscission. In one experiment (**3**) it was found that weekly spraying with 4-chlorophenoxyacetate decreased both relative fruitfulness and the number of bolls per plant; beta-naphthoxyacetate increased significantly the number of bolls set but, like naphthaleneacetate, was without significant effect on relative fruitfulness. In the abscission of bean leaves in has been found (**1**) that ethylene treatment leads to rapid disintegration in the abscission zone, whereas 2,4-dichlorophenoxyacetic acid delays all anatomical changes. Going further, several investigators (**13, 14, 18**) have developed the idea that the ethylene-auxin balance is the critical relation in leaf abscission.

It is not easy to conclude when a subject such as the foregoing has been adequately investigated or when conclusions are sufficiently well supported. In the light of the literature, as here briefly reviewed, and the interpretations provided by the experimental data presented, it would seem, nevertheless, that the view often held that the cotton plant sets only as many bolls as it can nourish (or more specifically in the present paper, as many as it can supply with carbohydrate and nitrogen) requires new or other evidence for

its support. It does not follow, however, if many more bolls were set (perhaps by auxin application) that the plant would then be able to maintain the necessary carbohydrate and nitrogen supply so that all could be brought to full development without a reduction in their size. As pointed out elsewhere it is common to find that the bolls constitute over 60% of the above ground dry weight of the cotton plant at the end of the summer; it would seem doubtful that this order of reproductive efficiency could be pushed a great deal higher. But between locations, years, and varieties substantial differences in relative fruitfulness are often indicated. Although the relative fruitfulness of small and large plants may be the same under a given environment, it still follows that the highest yields result under the nutritional conditions that give the largest plants. Plants that have lost their floral buds through insect injury are commonly found to be rank-growing and unproductive; they may set a top crop after insect control but still remain relatively unfruitful due to the self-shading of so much of the foliage.

Summary

The adequacy of the nutritional interpretation of boll shedding in cotton has been re-examined with special reference to carbohydrate and nitrogen relations. The investigation included chemical analyses of plants that were girdled and those sprayed with sucrose and urea in various combinations. The seasonal march of carbohydrates and nitrogen in leaves, stems and bolls 13 days old and the shedding of bolls were followed.

Spraying the upper leaf surface of plants daily through the fruiting period with 20% sucrose, 1% nitrogen (urea) and the two in combination failed to improve growth and resulted in large significant decreases in the number of bolls that were set. Flowering rates were not affected. Sucrose was not accumulated. Nitrogen was absorbed and resulted in much lower sugar and starch concentrations in all tissues. The urea when used alone burned the margins of the leaves severely but not when it was combined with sucrose; the sucrose when combined with urea greatly reduced nitrogen accumulation.

In an experiment in August when control plants were shedding over half of their bolls, girdling, 2% urea spray (six times) and the two together caused large increases in boll shedding. The girdling increased carbohydrate levels, and the urea spray increased nitrogen and reduced carbohydrate; the two in combination increased both.

The carbohydrate levels in the middle third of cotton stems increased from mid-July, when nearly all bolls were being retained to mid-August when most of them were being shed. In mid-July, the carbohydrate concentrations in bolls 13 days old were much higher than in the stems but by mid-August the two were much alike. This evidence of failure of polar transport to the bolls as the plants become older did not provide an explanation of boll shedding; bolls of first, second and third plantings (the first plantings shedding most of their bolls and the latter retaining their bolls) when sampled in mid-August all had similar concentrations of carbohydrates and nitrogen.

It is concluded that the nutritional theory of boll shedding is poorly supported at least as regards carbohydrate and nitrogen relations. Even though the relative number of bolls carried by a cotton plant, or the percentage of bolls shed, is apparently not influenced by the level of carbohydrate or nitrogen supply, the absolute number it may develop is so affected. Within varieties and environments, the number of bolls per 100 grams of fresh stems and leaves remains rather constant even though nutritional factors may cause marked differences in plant growth. This relationship has suggested that boll shedding is controlled by the balance between auxin produced in the leaves and an anti-auxin or inhibitory material from developing bolls that is moved out into the fruiting branches.

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THE CLIMACTERIC RISE IN FRUIT RESPIRATION AS CONTROLLED BY PHOSPHORYLATIVE COUPLING

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Introduction

The ripening of fruit is accompanied by a group of physiological and chemical changes which are characteristic and moderately uniform as between different species of plants. Typical components of the ripening process are pectic transformations which lead to fruit softening, changes in fruit color, often with the disappearance of pigment such as chlorophyll and the appearance of new secondary pigments, changes in compounds responsible for taste or odor, transformation of reserve materials such as polysaccharides to simpler sugars, and finally, changes in the magnitude of the respiratory gas exchange. The respiration of many fruits after removal from the plant follows the general course shown for the avocado in figure 1 (**1**). Respiration drops slowly to a low point known as the preclimacteric minimum. At this point the fruit is still unripe. With the initiation of fruit ripening, rate of respiration rises from the preclimacteric minimum to the climacteric maximum. Edible maturity of the fruit coincides in many cases with the climacteric maximum in respiration, or follows this maximum by a few hours or days. Subsequent to the climacteric the respiratory rate drops steadily as the fruit passes into senescence.

We have tried to get some insight into the process of fruit ripening by study of the factors which underlie the changing rate of respiration during the climacteric cycle. The sudden rise in respiration from the preclimacteric minimum to the climacteric maximum should become understandable if one could discover the factor or factors which limit respiratory rate at the preclimacteric minimum. We have therefore studied the factors which limit respiratory rate, and in particular, the factors which might by alteration or increase bring about increases in rate of respiration of preclimacteric fruit.

Materials and methods

The study reported here has been concerned with avocado fruits of the variety Fuerte. The avocado was selected for the purpose because of the rapidity and uniformity of its climacteric cycle. These fruits remain in the preclimacteric state so long as they are attached to the tree. On removal from the tree they show a slowly decreasing rate of respiration, a decrease

which continues for approximately three days at 15° C. At this time the preclimacteric minimum is reached. Respiration then increases rapidly in rate for a further five days at which time the climacteric maximum is obtained. It is of importance for such experiments that fruits of known climacteric status be used. The exact position of each individual fruit on the climacteric curve (fig. 1) was therefore determined by following the rate of respiration of individual fruits, using a continuous flow method with the Beckman oxygen analyzer (**11**). In this way fruits within a few hours of the preclimacteric minimum or the climacteric maximum could be selected for use in initial experiments. For many experiments, however, fruits were held in the preclimacteric condition simply by placing the freshly picked fruit at 5 to 8° C and using them within a few days. Similarly, fruit in the climacteric condition were obtained by allowing ripening to take place at 25° C for five to six days.

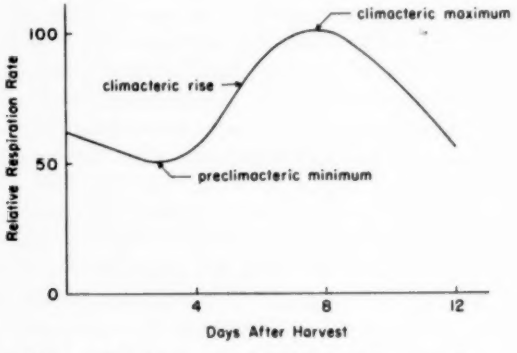

FIG. 1. Respiratory characteristics of the avocado.

Experiments with excised tissue were done with disks 1 cm. in diameter and 0.5 to 0.75 mm. thick. For this purpose a cork borer was used to remove a cylinder of fruit tissue which was then sliced with a hand microtome.

The isolation of particles capable of carrying on active respiratory metabolism was done by techniques similar to those used by LATIES (**4**) with cauliflower and by MILLERD *et al.* (**7**) with mung bean. The fruit was peeled, grated with a kitchen grater, and ground with quartz sand in 0.5 M sucrose solution. Thirty grams of tissue were ground in 50 ml. of the grinding solution. After a preliminary centrifugation at 2,000 × g for five minutes, the supernatant was centrifuged for 15 min. at 10,000 to 16,000 × g. The supernatant, including the fat accumulated at the surface, was removed by suction and the residue resuspended in 20 ml. of 0.5 M sucrose. This suspension was again centrifuged at 10,000 to 16,000 × g, the supernatant removed and the residue taken up in 3.0 ml. of 0.5 M sucrose. These operations, which are summarized in figure 2, were all carried out at 0 to 4° C. The enzymatically active particles isolated by this procedure have been

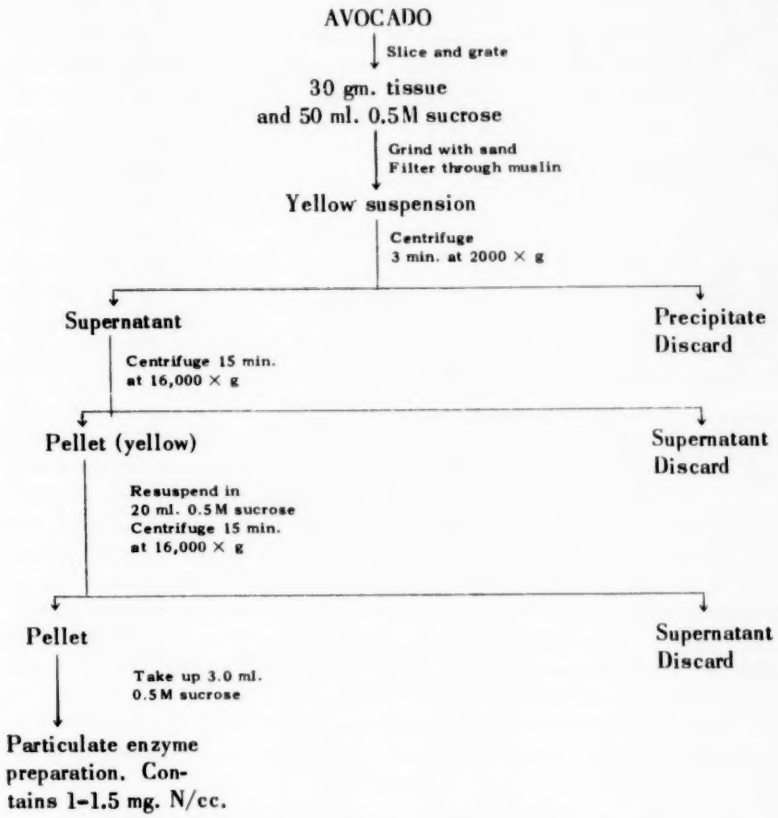

FIG. 2. Outline of procedures used in preparation of respiratory particles from avocado fruit.

shown to be similar to or identical with mitochondria (**7**) and will for convenience be termed mitochondria below, although no critical evidence is yet available on this point for the avocado.

Measurements of oxygen uptake of slices were measured by standard manometric procedures and at 25° C while measurements of enzymatic activity were made at 20° C.

Results

MITOCHONDRIAL OXIDATION OF PRECLIMACTERIC AND CLIMACTERIC FRUIT

The respiratory machinery of the avocado, as of other plant tissues which have been studied in detail, appears to be mediated by a particulate enzyme complex, a complex located in the mitochondria and competent to oxidize the acids of the Krebs cycle, including pyruvate. It is possible to prepare isolated mitochondria possessed of respiratory activity both from

TABLE I

OXIDATION OF ACIDS OF THE KREBS CYCLE BY CYTOPLASMIC PARTICLES OF PRECLIMACTERIC AVOCADO FRUIT. THE COMPLETE SYSTEM CONTAINS ENZYME, SUCROSE 0.3 M, DEXTROSE, 0.01 M, PHOSPHATE BUFFER, PH 7.1, 0.01 M, ADENOSINE-5-PHOSPHATE (AMP) 10^{-3} M AND $MgSO_4$ 10^{-3} M.

Substrate	Concentration	O_2 consumption
		mm.³ O_2/30 min./mg. N
Citrate	2×10^{-2} M	152
α-Ketoglutarate	2×10^{-2} M	125
Succinate	2×10^{-2} M	169
l-Malate	2×10^{-2} M	47
l-Malate	5×10^{-4} M	10
Pyruvate *	2×10^{-2} M	45

*Also contains l-Malate 5×10^{-4} M.

preclimacteric and climacteric avocado fruit. The data in table I show that respiratory particles prepared from the avocado are indeed able to oxidize the Krebs cycle acids at rates which are comparable to those obtained with respiratory particles of other plants. The respiratory particles or mitochondria prepared from preclimacteric fruit, are fully as active as those prepared from climacteric fruits. The data of table II suggest, in fact, that per unit of mitochondrial nitrogen, the mitochondria of preclimacteric fruits are even more active under the conditions of the experiments of table II than are the mitochondria of climacteric fruits. There is no reason to suppose that limitation in the amount of enzyme system itself is responsible for the low respiratory rate of preclimacteric fruits.

With all of the plant mitochondria, as well as with the animal mitochondria studied to date, oxidation of substrate is linked to the uptake of

TABLE II

OXIDATIVE AND PHOSPHORYLATIVE PROPERTIES OF THE MITOCHONDRIAL SYSTEM OF AVOCADO FRUITS. THE COMPLETE SYSTEM CONTAINS ENZYME, SUCROSE 0.3 M, DEXTROSE 0.01 M, PHOSPHATE BUFFER PH 7.1 0.01 M, AMP 10^{-3} M, $MgSO_4$ 10^{-3} M, NaF 0.01 M AND α-KETOGLUTARATE 0.02 M. TOTAL VOLUME 1.5 ML. ENZYME N/ML., PRECLIMACTERIC: 0.64 MG./ML., CLIMACTERIC: 0.76 MG./ML. REACTION MIXTURE. TEMPERATURE 20°C. ONE MILLILITER OF REACTION MIXTURE CONTAINS ENZYME FROM 3.3 GM. FRESH WEIGHT OF FRUIT. ALL CHEMICAL TRANSFORMATIONS ARE EXPRESSED IN TERMS OF MICROATOMS PER ML. OF REACTION MIXTURE.

Source of tissue	System	Uptake of phosphorus	Uptake of oxygen	Ratio of phosphorus to oxygen
		μ atoms/ml./30 min.		
Preclimacteric fruit	Complete	1.46	2.17	0.68
	No adenylate	0.12	0.37	0.32
Climacteric fruit	Complete	1.09	1.08	0.99
	No adenylate	0.13	0.40	0.33

inorganic phosphate and incorporation of this material into adenosine triphosphate (ATP) (**3, 6**). Rate of mitochondrial oxidation is therefore limited by availability of phosphate acceptor. The data of table II show not only that mitochondria from both preclimacteric and climacteric avocados possess the ability to carry on such coupled phosphorylation, but show also that the rate of oxidation by such mitochondria depends upon the presence of a phosphate acceptor such as adenylate. In the absence of suitable phosphate acceptor, the oxidative rate of avocado mitochondria is from one half to one fifth that found in the complete system. The experimental system for the demonstration of coupled phosphorylation with the respiratory particles of avocado (table II) is similar to that which has been used with other plant material (**3**). Inorganic phosphate is transformed in the course of coupled phosphorylation into the phosphate of ATP. This ATP is subject to degradation by adenosine triphosphatase which is present in avocado

TABLE III

EFFECT OF DNP ON THE RATE OF α-KETOGLUTARATE OXIDATION BY MITOCHONDRIA OF PRECLIMACTERIC AVOCADO. RATES ARE BASED ON THE FIRST 10 MINUTES. THE REACTION MIXTURE CONTAINS ENZYME, SUCROSE 0.3 M, DEXTROSE 0.01 M, PHOSPHATE BUFFER PH 7.1 0.01 M, $MgSO_4$ 10^{-3} M, NaF 0.01 M AND α-KETOGLUTARATE 0.02 M. TOTAL VOLUME 1.5 ML. ENZYME N 1.28 MG./ML. REACTION MIXTURE. TEMPERATURE 20°C.

Adenylate concentration	DNP concentration	Oxygen uptake	Increase by DNP
		mm.³/ml./30 min.	%
None	None	38	....
	10^{-5} M	59	55
10^{-4} M	None	78	....
	10^{-5} M	92	18
10^{-3} M	None	86	....
	10^{-5} M	92	7

as in other mitochondria (**3, 7**). The action of this enzyme is however minimized by the fluoride (0.01 M NaF) present in the reaction mixture. The ATP is also subject to attack by hexokinase which is present in avocado as in other plant mitochondria (**7, 9**) and which transfers the terminal phosphate to glucose (0.01 M in the reaction mixture) to form the stable glucose-6-phosphate. Thus as oxidative phosphorylation proceeds in this system, inorganic phosphate is transformed to glucose-6-phosphate. The extent of oxidative phosphorylation is measured simply by estimation of the residual inorganic phosphate of the reaction mixture.

It is known from work of recent years that the coupling of mitochondrial oxidations to oxidative phosphorylation can be severed by certain reagents, including 2,4-dinitrophenol (DNP) (**3, 5, 10**). By decreasing or abolishing the requirement for exogenous adenylate, DNP frees the mitochondria from dependence on this material and permits rapid mitochondrial oxidation to occur even in the presence of low adenylate concentrations. Table III

shows that dinitrophenol greatly increases the rate of oxidation of avocado mitochondria in the absence of adenylate. With these mitochondria, as with those of other tissues, dinitrophenol also decreases or abolishes the oxidative incorporation of phosphate into organic form (fig. 3). The effects of DNP with mitochondria of avocado as with other mitochondria are therefore two-fold and consist both in emancipation of the respiratory oxidation from the adenylate requirement and in suppression of the normal energy transfer from respiration through ATP.

Factors limiting respiratory rate in tissue slices

Dinitrophenol is not only effective *in vitro* but also may be used *in vivo* as a reagent for assessing the extent to which respiratory rate of a tissue is limited by the phosphorylative system (**2, 8**). In tissues which are limited

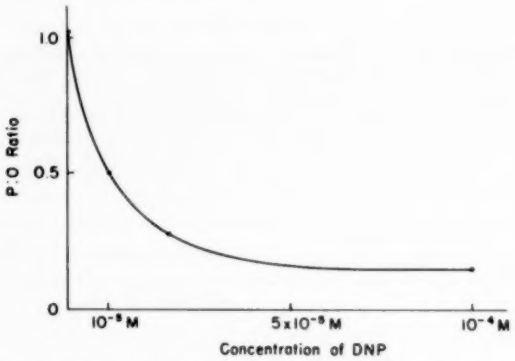

Fig. 3. Effect of dinitrophenol (DNP) on phosphorylative efficiency of α-ketoglutarate oxidation by avocado mitochondria. The complete system contains enzyme, sucrose 0.3 M, dextrose 0.01 M, phosphate buffer pH 7.1 0.01 M, AMP 10^{-3} M, $MgSO_4$ 10^{-3} M, NaF 0.01 M and α-ketoglutarate 0.02 M. Total volume 1.5 ml. Enzyme N 0.59 mg./ml. reaction mixture. Temperature, 20° C.

by the amount of adenylate, and hence, by capacity of the phosphorylative system, dinitrophenol brings about large increases in respiration. In tissues which are not limited by adenylate, but are limited by other matters, such as substrate, *etc.*, dinitrophenol is without such an effect on respiratory rate. The data of table IV show that dinitrophenol exerts a marked effect on the rate of respiration of avocado slices provided only that these slices are taken from preclimacteric fruit. With slices taken from climacteric or senescent fruit, on the contrary, dinitrophenol does not increase respiratory rate. It is noteworthy, however, that the climacteric rise in respiratory rate is not nearly as marked when measured with slices as it is with the intact fruit from which the slices are made. The slices respire more rapidly than an equal amount of tissue in the intact fruit, and the difference is more marked for the preclimacteric than for the climacteric fruit. The basis of

this behavior is still unknown. It does appear from this experiment, however, that the climacteric rise in respiration in the avocado is in some manner associated with changes in the phosphorylative coupling system. The phosphorylative coupling system appears to limit respiratory rate in tissue from the preclimacteric fruit but does not limit respiratory rate in tissues from fruit at the climacteric maximum.

Native uncoupling agents

Let us now suppose that the normal climacteric rise in respiration is indeed to be attributed to the removal of a limitation previously imposed by the capacity of the phosphorylative system. In what ways might removal of this limitation be achieved in the ripening fruit? One possibility

TABLE IV

EFFECT OF DNP ON THE RESPIRATION OF SLICES MADE FROM PRECLIMACTERIC AVOCADOS AND SLICES MADE FROM CLIMACTERIC AVOCADOS. TEMPERATURE 25°C.

Stage of fruit used as source of tissue	Number of measurements	O_2 uptake of whole fruit	O_2 uptake of slices				
			Control	DNP 10^{-5} M	Per cent. increase	DNP 10^{-4} M	Per cent. increase
		mm.³ O_2/gm./hr.	*mm.³/mg. dry wt./hr.*				
Fresh from tree	5	....	0.64	1.12	75	1.42	122
At climacteric minimum	7	25	0.68	1.11	63	1.36	100
On climacteric rise	1	72	0.73	0.90	23	0.85	16
At climacteric peak	6	93	0.60	0.59	−2	0.55	−8

is that during the ripening process, additional amounts of adenylate might appear within the fruit, or alternatively, that ripening involves the expenditure of energy and that utilization and turnover of adenylate is thus increased. A second general possibility would evidently involve the production during ripening of materials which, like DNP, would decrease the dependence of fruit respiration on the phosphorylative system. The implications of these two alternatives for our understanding of the process of fruit ripening are considerable. According to the first alternative, ripening should be regarded as a positive process involving the doing of some sort of biological work. The increased rate of respiration in ripening fruit would be a measure of the increased rate of utilization of ATP in the execution of this work. According to the second view, on the other hand, fruit ripening would consist of a generally degenerative process, and would be based on

TABLE V

EFFECT OF A SUBSTANCE OR SUBSTANCES FROM CLIMACTERIC AVOCADOS ON RATE OF MITOCHONDRIAL OXIDATION AND OXIDATIVE PHOSPHORYLATION. THE TEST SYSTEM IS MITOCHONDRIA OF MUNG BEAN. THE COMPLETE SYSTEM CONTAINS ENZYME, SUCROSE 0.3 M, DEXTROSE 0.01 M, PHOSPHATE BUFFER PH 7.1 0.01 M, AMP 10^{-3} M, $MgSO_4$ 10^{-3} M, NaF 0.01 M AND α-KETOGLUTARATE 0.02 M. TOTAL VOLUME 1.5 ML. ENZYME N 0.32 MG./ML. REACTION MIXTURE. TEMPERATURE 20°C. SUPERNATANT WHERE USED WAS ADDED AT THE RATE OF 1 ML. PER 3 ML. OF TOTAL REACTION MIXTURE.

System	Added supernatant from	Uptake of phosphorus	Uptake of oxygen	Ratio of phosphorus to oxygen
		μ atoms/ml./30 min.		
Complete	None	1.16	1.23	0.94
No adenylate	None	0.11	0.35	0.31
No adenylate	Climacteric fruit	0.06	1.25	0.05
No adenylate	Preclimacteric fruit	0.04	0.30	0.13

the dissociation of respiration from phosphorylation and energy transfer. According to this view fruit ripening would result from diminution of the ATP supply to a level inadequate to maintain the metabolic level characteristic of the unripe fruit. The evidence to be presented below suggests that the latter alternative is the more plausible and that fruit ripening, in the avocado at least, may perhaps be regarded as an uncoupling process.

That substances which are capable of replacing or supplanting adenylate in increasing rate of mitochondrial oxidation are produced in avocado fruit during ripening is shown in the data of table V. These substances are found in the supernatant which remains after the removal of the mitochondria by centrifugation at 16,000 × gravity. Their effects may be demonstrated with

TABLE VI

PROPERTIES OF SUBSTANCES IN CLIMACTERIC AVOCADO SUPERNATANT ON OXIDATION AND PHOSPHORYLATION BY MUNG BEAN MITOCHONDRIA. THE COMPLETE SYSTEM CONTAINS ENZYME, SUCROSE 0.3 M, DEXTROSE 0.01 M, PHOSPHATE BUFFER 0.01 M, AMP 10^{-3} M, $MgSO_4$ 10^{-3} M, NaF 0.01 M AND α-KETOGLUTARATE 0.02 M. TOTAL VOLUME 1.5 ML. ENZYME N 0.32 MG./ML. REACTION MIXTURE. TEMPERATURE 20°C.

System	Supernatant	Uptake of phosphorus	Uptake of oxygen	Ratio of phosphorus to oxygen
		μ atoms/ml./30 min.		
Complete	None	1.16	1.23	0.94
No adenylate	None	0.11	0.35	0.31
No adenylate	Climacteric fruit	0.06	1.25	0.05
No adenylate	Same heated	0.48	0.76	0.63
No adenylate *	Same dialyzed	0.22	1.34	0.16

* Data from comparable but separate experiment.

mitochondria of either preclimacteric or climacteric fruit or alternatively with unrelated mitochondria. The data in tables V and VI refer to the effects of avocado supernatants on the mitochondrial system of mung bean, a system which has been extensively studied and standardized (**3, 6, 7**). It is clear that the supernatant of the climacteric fruit causes a greatly increased oxygen uptake in the adenylate deficient mitochondrial system. The supernatant can evoke maximum oxidative rate from mung bean mitochondria even in the absence of exogenous adenylate. Phosphorylation is at the same time essentially totally suppressed. These effects are not exerted or are exerted to only a slight degree by the supernatant from preclimacteric fruits. Results entirely similar to those of table V were obtained in experiments in which supernatants of preclimacteric and climacteric avocados were supplied to avocado rather than mung bean mitochondria.

The data of table V clearly suggest that mature avocado fruits contain a principle or principles which like DNP act to decrease the degree of the dependence of respiratory oxidation on coupled phosphorylation. Further information on the nature of this principle is contained in the data of table VI. The striking effect of the supernatant of ripe avocados on the rate of mitochondrial oxidation appears to be due to at least two materials. The first is heat stable and acts qualitatively like adenylate itself. This factor alone causes a modest increase in oxygen uptake coupled with an appreciable phosphorylation. It is to noted that this factor is found in preclimacteric as well as in climacteric fruit. The second factor is heat labile and nondialyzable and may well be a protein. To this factor may be attributed the bulk of the supernatant effect. An attractive although hypothetical possibility is that the uncoupling may be owing to a soluble ATPase, since ATPase activity like the uncoupling activity develops in the avocado during the course of ripening.

Discussion

The evidence presented above suggests that respiratory rate in the unripe avocado is limited by the capacity of the phosphorylative system; by availability of acceptor for organic phosphate produced during oxidative phosphorylation. As the respiratory rate of the fruit rises from the preclimacteric minimum to the climacteric maximum, this limitation is gradually removed. A preliminary investigation of the factors involved has shown that the ripe, but not the unripe, fruit contains soluble materials which possess the ability to uncouple respiratory oxidations from their associated phosphorylative systems. These facts taken together suggest that ripening in the avocado may be in fact an uncoupling process and that the varied chemical changes which accompany ripening may result from this uncoupling. It will be evident that a critical test of this hypothesis may be based on determination of whether or not known uncoupling agents such as DNP are themselves able to induce the chemical changes characteristic of ripening.

Summary

This work concerns the climacteric rise in respiration of ripening fruit. The characteristics of the respiration of preclimacteric fruit have been contrasted with those of climacteric fruit. The respiration of the avocado fruit appears to be mediated by an organized respiratory complex. The active respiratory particles have been isolated from both preclimacteric and climacteric fruit.

The oxidation of substrate by the respiratory particles of avocado is coupled to the oxidative production of ATP. Rate of substrate oxidation by the respiratory particles is dependent upon the concentration of exogenous phosphate acceptor; adenylate. Although rate of substrate oxidation is normally limited by the capacity of the phosphate accepting system, this limitation can be removed by agents such as DNP which sever the coupling of oxidation to phosphorylation.

With the aid of the uncoupling agent, DNP, it has been shown that the rate of respiration of the preclimacteric but not of the climacteric fruit is limited by the capacity of the phosphate transfer system. The fact that ripening avocado fruits contain a substance which like DNP acts as an uncoupling agent suggests that both the climacteric rise in respiration and the chemical changes of fruit ripening may be owing to uncoupling: a decrease in synthesis or availability of ATP within the fruit.

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BRIEF PAPERS

DISTRIBUTION OF TOTAL SOLUBLE SOLIDS, ASCORBIC ACID, TOTAL ACID, AND BROMELIN ACTIVITY IN THE FRUIT OF THE NATAL PINEAPPLE (*ANANAS COMOSUS* L. MERR.)

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During the course of investigations on the cause of physiological breakdown in the fruits of the pineapple (*Ananas comosus* L. Merr.) it was observed that symptoms of this disorder are usually more pronounced in the lower half or lower two thirds of the fruit than in the region just below the crown. It, therefore, seemed desirable to make detailed studies of the distribution of some of the juice constituents of the pineapple, for it appeared that there may be physiological differences between top and bottom segments of the fruit.

The idea of unequal distribution of juice constituents in fruits is not new. BARTHOLOMEW and SINCLAIR (**4**) reported that mature oranges and grapefruits had a considerably higher concentration of total soluble solids in the stylar than in the stem-ends. LALL (**6**) and ARCHBOLD and BARTER (**1**) found that the concentration of soluble solids was higher in the blushed than in the unblushed side of an apple, and that these constituents increased from the stalk-end to blossom-end and from the inside to outside. The latter two investigators noted also that acid content of apples increased from outside to inside of the fruits. According to TUCKER (**10**), soluble solids in the watermelon increase progressively from the rind to the core, the greatest concentration being in the area around the seeds. Ascorbic acid has been reported higher in the peel than in the flesh of citrus fruits (**2**), apples (**5**) and tomatoes (**11**). SIDERIS *et al.* (**8**), in studies of pineapple fruits, found slightly greater amounts of soluble solids and less acid in the flesh than in the shell of the fruits. Apices of both shell and flesh were higher in acid and lower in soluble solids than the bases.

Natal pineapples in the mature green stage of maturity were shipped by railway express from Miami, Florida to Pittsburgh, Pennsylvania. The fruits were ripened at room temperature (25 to 30° C) before sampling. Pineapples were peeled, cored, and then cut into three transverse sections, *i.e.*, top, middle, and bottom. Methods employed for extraction of juice and determination of total soluble solids, total and ascorbic acids were identical with those described elsewhere (**7**). Bromelin activity was estimated by a method which has been recommended by BALLS and HOOVER for quantitative

determination of papain (**3**). Thirty pineapples were employed in this study, each fruit being analyzed separately.

Results of the analyses of 30 individual fruits have been averaged and are presented in table I. It will be observed that total soluble solids were higher in the basal than in the middle segments and higher in the latter than in the top portions. Total and ascorbic acids and bromelin activity were highest in the top segments and decreased progressively toward the base of the pineapples.

As a rule, ripening of fruits is accompanied by an increase in soluble solids, a decrease in total acid and in some instances by a decrease in ascorbic acid. The results now being reported indicate that in the so-called mature pineapple, the basal segment is more mature than the middle portion, and this in turn is more mature than the top segment. This result is also confirmed by the progressive decrease in bromelin activity from top to bottom since it has been reported by TANAKA (**9**) that activity of this enzyme tends to decrease with increasing maturity of the pineapple fruit. It is apparent, therefore, that physiological breakdown in pineapples is associated with physiological maturity and that the disorder is similar to breakdown in a number of other fruits in this respect. This observation is borne out by the fact that pineapples which have been chilled while green may not show definite symptoms of physiological breakdown until they become ripe.

TABLE I

WEIGHT, JUICE VOLUME AND DISTRIBUTION OF VARIOUS JUICE CONSTITUENTS IN THREE TRANSVERSE SECTIONS OF THE FRUIT OF THE NATAL PINEAPPLE (AVERAGE OF 30 FRUITS).

Section	Weight	Volume of juice	Total soluble solids	Total acid	Ascorbic acid	Bromelin activity
	gm.	*ml.*	*%*	*gm./100 ml.*	*mg./100 ml.*	*1/t*
Top	160	72	14.7	1.22	28.4	0.539
Middle	202	89	17.1	1.15	21.7	0.497
Bottom	194	83	19.3	1.10	13.2	0.446

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THE COMPOSITION OF PLANT FRACTIONS EXTRACTED WITH 80% ALCOHOL

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In the extraction of plant products with 80% ethyl alcohol varying lengths of time have been used with little consideration of the relationship between length of time and completeness of extraction. LOOMIS and SHULL (**4**) state that one should extract plant material until no further pigments or significant quantities of sugar or nitrogen could be detected. JOSLYN and STEPKA (**3**) extracted amino acids by grinding the material in a Waring Blendor for five minutes, allowing the minced sample to stand overnight at room temperature, and then storing for about one week in alcohol at 0° C. GIBBS (**1**) obtained sugars and amino acids by extracting sunflower seeds with 80% ethyl alcohol for 20 minutes. While the length of time necessary for completeness of extraction may vary from one plant material to another, information in this regard obtained under a standard set of conditions would be of great value. This is especially true for studies involving radioactive isotopes where prolonged solvent extraction might be accompanied by further loss of radioactivity.

Shoots of young corn plants which had been exposed to light and to $C^{14}O_2$ for 15 hours under greenhouse conditions were used in this study. A sample of fresh plant material weighing 8.7 gm. (about 0.87 gm. dry weight) having an initial pH of 5.7 was cut into small pieces and extracted with 150 ml. of hot 80% ethyl alcohol until most of the pigments were removed. The solvent was then decanted, and the tissue was extracted with 100 ml. of fresh 80% alcohol in a Soxhlet apparatus which siphoned every three minutes. At intervals, the extract was removed from the receiving flask and replaced with fresh solvent. Each extract was filtered, diluted with an equal volume of water, and treated with ethyl ether to remove pigments and lipids. The ether soluble fraction was termed the ether extract and the remainder was called the residual alcohol extract. These extracts were evaporated to dryness and weighed. Radioactivity measurements of these residues were then made with a Geiger-Muller counter.

The residual alcohol extract was then redissolved in ethyl alcohol and its final volume adjusted to one milliliter. The following groups of substances were detected by partition paper chromatography: organic acids, amino acids, and sugars. Solvents for the organic acids were water-saturated phenol and a mixture of reagents of the following proportions: 1 ml. isopropyl alcohol, 3 ml. tertiary butyl alcohol, 3 ml. benzyl alcohol, and 1 ml. water with 1% of the final volume being 90% formic acid. Bromcresol

green was used as the color developing agent. Solvents for amino acid chromatography were water-saturated phenol, and equal quantities of water-saturated 2-3-6 collidine and 2-6 lutidine, using ninhydrin as the color-developing agent. The sugar chromatography solvents were water-saturated phenol and a mixture of reagents of the following proportions: 4 ml. butanol, 1 ml. ethanol, and 1 ml. water. 3,5-dinitrosalicylate reagent was used as a color-developing agent, after the method of JEANES (**2**).

At least four paper chromatograms were made for the detection of each class of substances. The identity and concentration of each of these substances was established by the visual density method and by comparison with chromatographic spots produced by pure substances under identical conditions.

TABLE I

RESULTS OF WEIGHT AND RADIOACTIVITY DETERMINATIONS OF MATERIALS OBTAINED FROM CORN SHOOTS BY CONTINUOUS EXTRACTION WITH 80% ETHYL ALCOHOL.

Extraction interval	Young corn shoots					
	Ether extract of 80% alcohol fraction			Residual alcohol extract		
	Weight	Radioactivity		Weight	Radioactivity	
hrs.	*mg.*	*counts per min.*	*counts per min. per mg.*	*mg.*	*counts per min.*	*counts per min. per mg.*
0–24	7.5	143	19	12.7	3,960	312
24–48	6.7	195	29	12.0	3,589	299
48–72	1.7	112	66	6.0	795	132
72–96	0.9	65	72	4.1	271	68
96–120	0.3	30	100	1.8	11	6
120–146	0.5	26	52	4.5	137	30
146–192	0.3	14	47	3.5	106	30
192–240	0.3	29	97	3.9	146	38
240–336	0.6	16	3	7.9	134	17
336–432	0.6	6	1	9.3	91	10
432–528	....	....	....	2.3	74	32
Total	18.9	636		68.4	9,314	

A modified Folin-Wu method was also used for the determination of total reducing sugars.

The analysis of the alcohol extract of young corn plants showed that extraction, under these specific conditions, was generally less than half complete at the end of 24 hours. The weights of ether extracts (table I) fell off more rapidly than weights of the residual alcohol extracts with time. Only 53% of the total extractable weight was removed during the first 72 hours. Thereafter, there was still a small but continued extraction of alcohol-soluble material. When the experiment was discontinued at the end of 528 hours, extraction was still not complete. The dry weight of the residue accumulated during the last 96 hours of extraction comprised nearly 3% of the total material extracted.

Table I shows that after the first 72 hours of extraction the level of radioactivity of the substances extracted decreased markedly though at a more or less constant rate. Substances containing radioactivity were extracted in significant quantities even during the last 96 hours of extraction. The specific radioactivity in counts per minute per milligram of dry weight of these extracts showed a pattern similar to that based on total counts per sample.

TABLE II

MATERIALS OBTAINED FROM CONTINUOUS EXTRACTION OF YOUNG CORN SHOOTS WITH 80% ETHYL ALCOHOL. AMOUNTS IN MG. ARE ESTIMATED FROM THE DENSITY PRODUCED ON PAPER CHROMATOGRAMS AND FROM A REDUCING SUGAR METHOD.

Materials	Extraction interval in hours			
	0–24	24–48	48–72	72–96
	mg.	*mg.*	*mg.*	*mg.*
Organic acids				
Aconitic	1.67	0.87	0.35	0.17
Adipic	0.46	0.29	0.12	0.02
Citric	0.09	0.77	0.38	0.08
Glycolic	0.01	0.01	....	....
Malic	0.34	0.27	0.08	0.01
Oxalic	0.61	0.13	0.01	....
Tartaric	0.27	1.00	0.34	0.34
Unidentified	0.22	0.01	....	....
Total	3.67	3.35	1.28	0.62
Amino acids				
Aspartic	0.08	0.12	0.06	0.03
Glutamic	0.30	0.06	0.06	0.01
Lysine	0.54	....	....	....
Methionine	trace	....	....	....
Serine	0.60	0.03	trace	....
Valine	0.34	0.01	....	....
Glutamine	0.31	....	....	....
Unidentified amino acids	+	+	....	....
Total	2.17	0.22	0.12	0.04
Sugars				
Reducing sugars	5.76	0.57	0.16	0.04
Glucose	3.79	0.18	0.04	....
Fructose	1.08	0.04	....	....
Cellobiose?	0.91	....	....	....
Sucrose	1.03	....	....	....
Raffinose	1.07	....	....	....
Total	7.88	0.22	0.04	....

The results of the chromatographic analysis of materials extracted from corn shoots by continuous extraction with 80% alcohol are given in table II. It was found that the composition of the materials extracted varied with different lengths of extraction time. Sucrose and raffinose, and a compound which had a position on the chromatogram similar to that of cellobiose, could not be detected after the first 24 hours of extraction. At the end of

72 hours of extraction no sugars could be detected by chromatography. However, the copper oxide method showed that small but significant amounts of reducing substances were extracted by this method even at 336 to 432 hours. These substances might have arisen from the hydrolysis of glucosides.

Most of the substances which appeared to be organic acids could not be detected by chromatography after 96 hours of extraction. A substance presumed by its location to be aconitic acid, however, could be detected even after 336 to 432 hours of extraction and tartaric acid after 192 to 240 hours. Further, chromatographic results suggested that more tartaric and citric acids were extracted during the 24- to 48-hour interval than during the first 24-hour extraction period. When the three columns of data for the residual alcohol extracts in table I are plotted on a cumulative basis against time, an extraction of the greatest quantity of radioactive material occurred between the 24- and 146-hour interval while the greatest radioactivity was found in the 24- to 48-hour interval for the ether extracts.

The fact that the sum of the components of the residual alcohol extract did not equal the weight of the whole residual alcohol extract (528-hour total) is probably a result of inability to detect these various compounds in such small quantities.

The experiment was repeated with corn plants and again with the grass, *Andropogon ischaemum* L. Results of these later experiments showed an extraction of most of the radioactive material in the first 72 hours. In short, the pattern of extraction of substances found in the corn shoots was found to hold for grass shoot samples also.

The writers recognize that the time of extraction for a particular class of substances must be the best compromise between speed and accuracy. These results do suggest, however, that there is a need for critical inspection of the time factor in the extraction of certain plant products with 80% ethyl alcohol. This is especially true of studies involving radioactive-labeled materials.

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NITRATE REDUCTION BY CHLORELLA

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During the course of metabolic studies with Chlorella it was found that oxygen was produced when cells were illuminated in modified Knop's solution containing glucose, without atmospheric carbon dioxide. This observation has a bearing on the problem of nitrate reduction by plants and forms the basis for this report.

Chlorella cells were grown on nutrient agar slants under continuous illumination of 140 fc at 23.5 ± 1.5° C. The medium contained: KNO_3, 1.21 gm.; $MgSO_4$, 1.20 gm.; KH_2PO_4, 1.22 gm.; A4 microelement solution (1), 1.0 ml.; iron solution, 1.0 ml.; tryptone, 4.5 gm.; yeast extract, 0.36 gm.; glucose, 10 gm.; agar, 10 gm.; distilled water, 998 ml. The iron solution supplied the medium with 10^{-5} M ferrous sulphate and 10^{-5} M potassium citrate.

Cells from cultures 11 days old were harvested, washed with distilled water, and suspended in the medium in which their ability to evolve oxygen was to be measured. The solution, subsequently referred to as a modified Knop's solution, contained the major salts, microelements, and iron in the concentrations listed above. This solution plus 1% glucose formed the basic medium for the following investigation. All solutions were adjusted to pH 5 with sodium hydroxide and hydrochloric acid.

Gas evolution was measured in ordinary conical Warburg vessels which were illuminated from below by seven 100-watt incandescent lamps. The light intensity on the bottoms of the vessels was about 1,300 fc, with less than 1% variation across the length of the tank. Each vessel contained 1.8 ml. of cell suspension in the main chamber and 0.2 ml. of 20% KOH in a side arm. The illuminated area of each suspension was about 7.1 $cm.^2$

Chlorella cells were suspended in 1% glucose-modified Knop's solution in the absence of atmospheric carbon dioxide and illuminated. Although initially little or no positive pressure change developed, the rate of oxygen production gradually increased, until after two or three hours it was fairly rapid. The data plotted in figure 1 A were obtained six hours after the beginning of illumination. In most of the following experiments the data plotted were obtained after two or three hours illumination, a long enough time to avoid the lag phase consistently found.

Since the cells were exposed to light but were not given carbon dioxide, photosynthesis should no more than compensate for respiration. Under these conditions, some other process must have been ultimately responsible

for the production of oxygen. The purpose of the following experiments was to establish the factors and process responsible for this extra oxygen.

The necessity of glucose for the oxygen production is indicated by figure 1 A. Cells in modified Knop's solution liberated only 6.2% as much oxygen as cells in glucose-modified Knop's solution. Some component or components of modified Knop's solution are also required (fig. 1 B). Although oxygen was produced when cells were illuminated in a glucose solution without salts it only amounted to 28.3% of that produced by cells given both glucose and salts.

To determine the components in the mineral nutrient solution responsible for the production of the extra oxygen, tests were made with the individual major salts in the presence of glucose (fig. 2 A). Cells in glucose-KNO_3

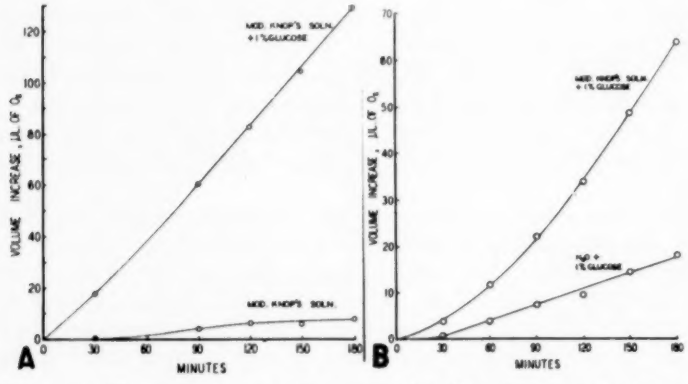

FIG. 1. A. The production of extra oxygen by Chlorella suspended in modified Knop's solution with and without glucose. Conditions: ca. 1,300 fc; KOH in side arm; 15.2 mm.³ cells/vessel; plot of data after six hours of illumination. B. The production of extra oxygen by Chlorella suspended in 1% glucose solution with and without nutrients of modified Knop's solution. Conditions: ca. 1,300 fc; KOH in side arm; 18.8 mm.³ cells/vessel; plot of data after two hours illumination.

solution produced 51.4% of the gas volume increase caused by cells in glucose-modified Knop's solution. Singly, KH_2PO_4 and $MgSO_4$ in the presence of glucose had no greater effect on oxygen production than glucose alone. From this it is concluded that KH_2PO_4 and $MgSO_4$ are not directly involved in the production of the extra oxygen, whereas KNO_3 is. Microelements and iron were tested singly and together with KNO_3 and glucose to determine whether they increase the production of extra oxygen (fig. 2 B). Combined they increased oxygen production only 12.5%, whereas an increase of 48.2% was necessary to equal the amount of extra oxygen produced by cells in glucose-modified Knop's solution. Next, combinations of the major salts with a mixture containing glucose, A4 microelements, and iron were tested. Of the major salts, KNO_3 in combination with KH_2PO_4 was necessary for the complete production of extra oxygen (fig. 3 A). The combination

$KNO_3 + MgSO_4$ was no more effective than KNO_3, while the combination $MgSO_4 + KH_2PO_4$ was no more effective than glucose would be alone.

It was demonstrated that live Chlorella cells are necessary for the production of extra oxygen. No pressure change was observed when cells killed by boiling were suspended in glucose-modified Knop's solution. Also, no pressure change occurred in vessels containing glucose-modified Knop's solution without cells, during experiments lasting 5 to 10 hours. The possibility that

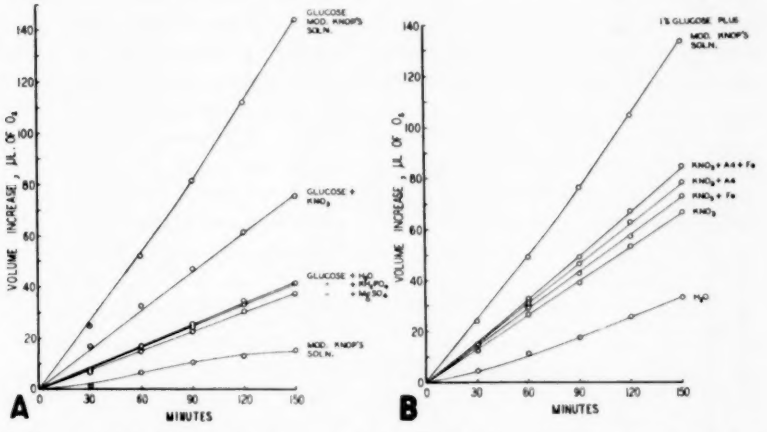

FIG. 2. A. Influence of the major salts in modified Knop's solution on the production of extra oxygen by Chlorella. Conditions: ca. 1,300 fc; KOH in side arm; plot of data after three hours of illumination. B. Influence of A4 microelements and iron on the production of extra oxygen by Chlorella supplied with glucose and KNO_3. Conditions: ca. 1,300 fc; KOH in side arm; 24 $mm.^3$ cells/vessel; plot of data after three hours of illumination.

the positive pressure changes were caused by contaminants giving off respiratory carbon dioxide which was caught by Chlorella and used for photosynthesis is thereby eliminated.

From the experiments reported it is apparent that three major constituents, glucose, nitrate, and phosphate, are necessary for the complete production of extra oxygen; and that the process ultimately responsible is nitrate reduction, for which glucose and phosphate ion are required.

If it is assumed that the process of nitrate reduction proceeds according to equation 1,

$$(1) \qquad KNO_3 + 2(CH_2O) \longrightarrow NH_3 + KOH + 2CO_2$$

$$(2) \qquad 2CO_2 + 2H_2O \xrightarrow{\text{light}} 2(CH_2O) + 2O_2$$

then for each mole of nitrate reduced, two moles of carbon dioxide would be produced and subsequently by photosynthesis (equation 2) two moles of oxygen would be liberated. To determine whether stoichiometric yields of oxygen are obtained according to these equations, cells were suspended

in media containing three concentrations of KNO_3. All of the media contained glucose, KH_2PO_4, microelements, and iron. The quantity of extra oxygen produced for a given amount of nitrate reduced was calculated as the difference between the amounts produced by cells with and without nitrate. Differences were not taken until after the slopes of the oxygen-liberation curves for the cells given nitrate became equal to the slope of the curve for the cells not given nitrate, which indicated that all of the nitrate had been reduced (fig. 3 B). The amount of extra oxygen produced for each quantity of nitrate reduced is given in table I. On the average, 109% of the theoretical yields were obtained which is added evidence that the process ultimately responsible for the production of extra oxygen is nitrate reduc-

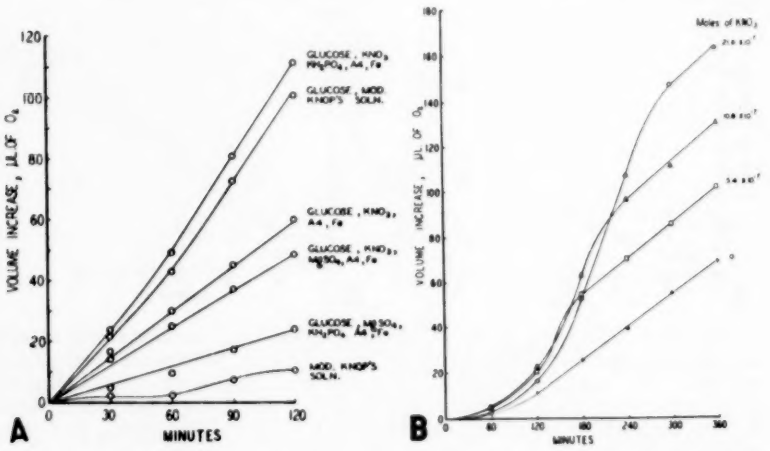

FIG. 3. A. Influence of combinations of the major salts in modified Knop's solution on the production of extra oxygen by Chlorella supplied with glucose, A4 microelements, and iron. Conditions: ca. 1,300 fc; KOH in side arm; 20 mm.^{3} cells/vessel; plot of data after three hours of illumination. B. Extra oxygen response of Chlorella to three concentrations of KNO_3. All of the media contained glucose, KH_2PO_4, A4 microelements, and iron. Conditions: ca. 1,300 fc; KOH in side arm; 25 mm.^{3} cells/vessel; plot of data from the beginning of illumination.

tion and makes the reactions of equations 1 and 2 a possible mechanism by which two moles of oxygen are produced per mole of nitrate reduced.

In light in the absence of atmospheric carbon dioxide, glucose and phosphate ion are required for the maximum rate of production of extra oxygen and concomitant nitrate reduction by Chlorella. The fact that both glucose and phosphate ion are necessary, strongly suggests that the process of nitrate reduction is coupled with the oxidation of glucose. There is little doubt that in darkness the hydrogen necessary for the reduction of nitrate is supplied by carbohydrate, but in light the problem is more complex because it is known that the rate of nitrate reduction in light is more rapid than it is in darkness. The problem of the mechanism of the light action is then presented.

TABLE I

YIELD OF EXTRA OXYGEN FOR NITRATE REDUCTION.

Moles of KNO_3 ($\times 10^{-7}$)	Extra oxygen				Per cent. theoretical yield
	With KNO_3 (1)	Without KNO_3 (2)	Yield (1 − 2)	Theoretical yield	
	$\mu l.$	$\mu l.$	$\mu l.$	$\mu l.$	
21.6	142.2	50.1	92.1	96.8	95.1
10.8	87.5	32.2	55.3	48.4	114.3
5.4	48.8	20.1	28.7	24.2	118.6
					Average 109.3

WARBURG and NEGELEIN (**8**) came to the conclusion that light accelerates the reduction by altering the absorbing mechanism so that more nitrate can penetrate the cell in a given time. The possibility nevertheless exists that light is directly involved by producing the reducing power via the photochemical reaction of photosynthesis (**7**). This photochemical reduction of nitrate can be written in the form of equation 3.

(3) $$HNO_3 + H_2O \xrightarrow{\text{light}} NH_3 + 2O_2$$

On the basis of the results reported in this paper it is not possible to assign to this reaction a substantial role in nitrate reduction, since cells in modified Knop's solution, without glucose, produced only a very slight amount of extra oxygen.

It might be argued that there would be no continuous reduction of nitrate without removal of the reduction products by assimilatory action of the organism. But the experiments of WARBURG and NEGELEIN (**8**) demonstrate that nitrate reduction by Chlorella continues even after the ammonia formed is no longer assimilated and accumulates in the medium. It has also been shown that even when carbohydrates are deficient in etiolated pea seedlings (**6**), Azotobacter (**4**), and *Aspergillus niger* and *Mucor racemosus* (**5**), these organisms continue to reduce nitrate. Recently KESSLER (**3**) was able to obtain the accumulation of nitrite in culture solutions of the green alga Ankistrodesmus by inhibiting the transformation of nitrite to ammonia by means of a low pH. These examples afford ample evidence that nitrate reduction continues even though the nitrite and ammonia formed are not assimilated.

It seems reasonable to conclude that if the photochemical reduction of nitrate, according to equation 3, plays a major role, then greater amounts of oxygen should have been produced by cells in modified Knop's solution without glucose. It is possible, of course, that the small amount of extra oxygen produced under such conditions was due to the photochemical reduction of nitrate. If this is so, then it is negligible compared with the reduction process requiring glucose. This small amount of extra oxygen, produced by cells in modified Knop's solution without glucose, may also have resulted from nitrate reduction for which carbohydrate reserves were used.

The ability of Chlorella when deprived of atmospheric carbon dioxide

and nutrient salts, but supplied with glucose, to liberate more oxygen than the amount necessary to compensate for respiration (figs. 1 B, 2 A, B and 3 B) is of particular interest. The rate of production of extra oxygen resulting from this reaction is approximately one sixth the rate of oxygen uptake during respiration with glucose as substrate. Presumably there is a mechanism, other than nitrate reduction, for the utilization of the reducing power resulting from photochemical reactions which does not rely upon the uptake of carbon dioxide. A possible mechanism is the reduction of intermediate oxidation products. This possibility is strengthened by evidence available on a photosynthetic Chlorella mutant unable to use atmospheric carbon dioxide for photosynthesis yet capable of producing oxygen in light (**2**). It is believed to accomplish this by reducing partially oxidized products of respiration.

The method of studying nitrate reduction by measuring the production of extra oxygen in light under conditions excluding carbon dioxide from the atmosphere is simple, relatively direct, and yields quantitative results. The long lag phase which was encountered is a disadvantage, but is not serious. It is possible that by working out appropriate methods this disadvantage can be overcome.

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BIOELECTRIC POTENTIALS OF TUMOR-INFECTED PLANTS

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Bioelectric potentials are considered by BURR (**2**) to be a vital part of life processes, and any change affecting life processes is bound to exert an effect on these potentials. There is little doubt that radiation injury exerts a profound effect on the potentials of the avian embryo, as shown by ROMANOFF and BLESS (**7**) and on seeds as found by BLESS (**1**) and JONES *et al.* (**6**). The effect of tumor on the potentials is not so unambiguous. BURR and coworkers (**3**) found changes in the potential, measured across the chest of mice with induced tumors, the character of the changes being dependent on the age and the strain of the animals. The work of BURROWS *et al.* (**4**) seems to contradict these findings. The object of these experiments is to study the effects of tumor on the potential of plants. Two sets of experiments separated by a considerable time interval have been performed with results that are essentially similar.

The circuit for measuring potentials was essentially a bridge network using an FP54 electrometer tube (**5**) *in vacuo* with an input resistance of 1.16×10^{11} ohms. With the voltages of the plants used the current was below 10^{-12} amperes. Contact with the plants was made by means of felt brushes moistened with a KCl solution flowing from test tubes into which were immersed Ag—AgCl electrodes. The sensitivity of the apparatus was about 0.1 mv./div.

Tomato plants were chosen for this study because of their relative homogeneity. Two groups of 30 plants each were cultivated; one group in six-inch standard flower pots, the other in four-inch pots. Fifteen of the plants in the six-inch together with 10 of the plants in the four-inch pots were maintained as control. All plants were inoculated; those in the experimental groups with a strain of *Phytomonas tumefaciens* which produces a hairy tumor, and those in the control groups with distilled water. The latter action was taken so that effects due to external injury would be the same for all plants. The injections were made with a hypodermic needle at a depth of about 1 mm. below the surface. Precautions were taken to be able to locate the exact spot of the inoculation. The plants were protected with bandages at the point of insertion of the needle to prevent further infection. The plants were approximately 50 cm. in height at the time of inoculation, and about 1 cm. in diameter.

The potential of each plant at the point of inoculation relative to a point directly across the stem was measured at the same time of day on alternate days. Resistance measurements were at first taken across the same portion

of the plant for which potential readings were taken, but these were discontinued when it was found that there was no apparent difference between the resistance of tumorous and normal tissues. The potential measurements for each of the four sub-groups were begun before inoculation and extended over a period of about five weeks.

The average potential of each of the four groups is plotted in figure 1. The potential reading of each plant was taken several times so that each point on the graph represents the average of as many as 80 readings.

As is usual with work on bioelectric potentials the readings varied within wide limits. The somewhat arbitrary elimination of 10% of the readings

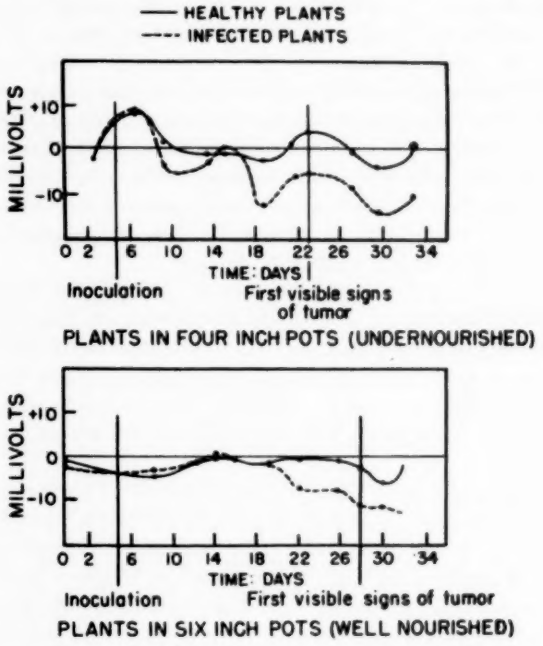

FIG. 1. Variation of potential of plants with time.

from each end did not change appreciably the average values but reduced considerably the deviations from the mean. The uncorrected average deviation was about 0.5 mv. while the standard deviation from the mean was about 10 times as large.

It is evident from the figure that the potentials of the infected plants in the larger pots were essentially the same as those of controls during the first 16 days after inoculation. After that the potentials of the infected plants were considerably lower than those of controls. Differences in the average of the two groups became evident about seven days before the tumors became visible. In the second series of experiments, readings of the

potential were taken daily and the point of inoculation was observed by means of a pocket microscope. The tumor became discernible in 30% of the plants at about the same time as significant differences of potential appeared in the two groups. The plants in both groups were all vigorous and well nourished, since they were cultivated in pots large enough to produce fruit.

The potentials of plants grown in smaller pots followed exactly the same pattern. At first the potentials in the two groups were similar. Later the infected group showed a lower average potential than the control, which became definitely noticeable about seven days before the appearance of the visible signs of the tumor. However, the tumor as well as the significantly lower potentials appeared four days earlier than in the larger pots.

The plants in the smaller pots were definitely undernourished, being cultivated in containers too small to allow fruiting without the addition of special nutrients. They were considerably smaller than those in the larger

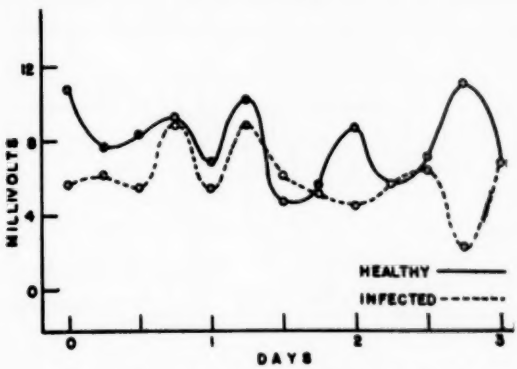

FIG. 2. Diurnal variation of potential of plants.

pots. The fact that the tumor in the plants grown in the smaller pots appeared earlier than in the larger pots indicates that the resistance of the plants to the tumor was lower in the undernourished group. This group also showed larger variation of potential than those in the larger pots.

The deviation of the mean from zero in the initial period of the experiment and of the mean of the uninfected plants throughout the experiment was attributed to injury produced by inoculation. Obviously readings taken directly across the stem of the uninfected and uninjured plants would probably tend to average zero, providing the readings are not taken near a node.

During the seven-day period before the appearance of the tumor, a few plants still showed a higher potential for the inoculated side. This exceptional and disappointing behavior proved to be due to the fact that for some reason the tumor appeared on the side of the stem opposite to that of inoculation. The side on which the tumor appeared was consistently negative with respect to the opposite point of the stem although the magnitude of the negativity varied.

The potentials of individual plants varied greatly at first in magnitude and sign, and later in magnitude only. It seemed interesting to find out whether the variation has a diurnal periodicity. The potentials of 10 plants from each of the infected and uninfected groups were measured at six-hour intervals for 72 hours. The measurements were started at a time when about 30% of the plants showed the presence of tumor when observed with a microscope. The variation of the average potential for each of the groups is shown in figure 2. As is evident from the figure there seems to be a pediodicity irregular in time in the case of the uninfected plants, while those infected seem to show a different periodicity.

Potential gradients were plotted for six plants cultivated in six-inch pots, three healthy and three infected, subsequent to inoculation but prior to the appearance of tumor. One contact was kept fixed at a point about 1 cm. above the level of the soil and the other was moved upward along the opposite side of the stem in 1 cm. intervals. The resulting curve is shown in figure 3. It was expected that the gradient would be affected by the pres-

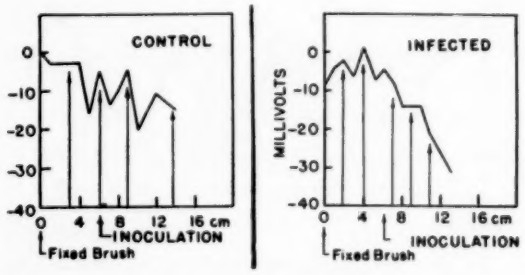

FIG. 3. Variation of potential of plants along the stem. Arrows point to nodes.

ence of the tumor in the infected plants, but no such behavior was found. Two interesting facts were revealed, however; the trend of the gradients seemed to be from the higher to lower potential upward along the stem, and the presence of leaf sprouts caused in most cases a sharp rise in the curve.

These experiments indicate that the bioelectric potentials are affected by the presence of a tumor in plants even before the tumor becomes visible to the unaided eye. While the potential of a plant fluctuates greatly during its growth, the point at which a tumor develops shows an unmistakable trend to become electronegative with respect to a neutral point.

The authors are indebted to Dr. Nathan Smith, senior bacteriologist of U.S.D.A. for the culture of Phytomonas used in these experiments. Part of this work was done under the auspices of the AEC.

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CAROTENOIDS IN GRAPEFRUIT, *CITRUS PARADISI*

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The grapefruit, *Citrus paradisi* Macf., is not well suited to genetic studies, but mutants show characteristic changes in the pigmentation of the fruit flesh. It seemed of interest therefore to analyze these changes to see whether they were in any way comparable with changes known to occur in the tomato fruit (**2**). The following sequence of events appears probable: the shaddock, or pummelo, *Citrus maxima* (Burm.) Merr., is considered to be the progenitor of the common Marsh White or colorless grapefruit of commerce (**1**). A mutant from Marsh White gave rise to fruit with a pink flesh, Marsh Pink. Finally bud sports of Marsh Pink gave rise (**1, 5**) to fruit deep pink in color, Ruby (syn. Texas Pink and Arizona Red Blush).

The fruit of shaddock has been shown by MATLACK (**3**) to contain lycopene. Shaddock fruit examined in Los Angeles and in Riverside were found to be as devoid of color as the Marsh White. One cannot therefore state whether the grapefruit was derived from pink or colorless shaddock stock. However, sporting of the white grapefruit resulted in appearance (or reappearance) of lycopene and β-carotene (Marsh Pink) and this was intensified by further sporting to Ruby.

Nucellar embryony (production of several saplings from a single seed) makes it difficult to trace the genetic differences, but the nature of the carotenoids and the proportions in which they are found in the three cases is of definite interest in searches for possible pathways in carotenoid biosynthesis.

All fruit was purchased fresh under the following trade names, indicative of locality: Marsh White—Coachella White (Coachella, California); Marsh Pink—Coachella Pink; Ruby—Texas Pink (Rio Grande Valley), Arizona Red Blush (Mesa, Arizona).

Depending on the color, between 100 and 500 gm. of the flesh was exhaustively extracted with acetone and the carotenoids were transferred to petroleum ether and chromatographed on magnesium oxide–Hyflo Super Cel columns, as previously described (**2**). The absolute amounts of the separated individual components were then calculated from the optical densities at the respective maxima, by use of specific absorption coefficients given by PORTER and LINCOLN (**4**). In the case of lycopene, it was convenient to compute its concentration from values at 503 and 480 mμ for the crude extract assuming a two-component mixture of lycopene and β-carotene.

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The absorption curves of the crude extracts in petroleum ether were clearly indicative of the differences in proportions of the individual carotenoids. The Ruby (both from Texas and Arizona) gave curves showing characteristic bands, with maxima ca. 503, 472, and 448 mμ; the shape of these curves approximated that required by a 1 : 1 mixture of lycopene and β-carotene. The lycopene band at 503 mμ was not discernible in the crude extract of Coachella Pink, which gave a three-banded spectrum at 478, 450, 425 mμ, indicative of the predominance of β-carotene. The spectrum for the crude extract of Coachella White showed a shelf ca. 445 mμ and three well-defined bands at 425, 400, and 380 mμ.

Iodine catalysis of these crude extracts gave no significant changes (Coachella White) or a slight paling (in the lycopene-containing samples) in the visible portion of the spectrum. The absence of detectable quantities of poly-cis forms was confirmed by chromatography. Quantitative results for the major components in these varieties are given in table I.

A total of 11 zones were resolved though they were not all present in each case. The three pink strains contained predominantly β-carotene and

TABLE I

PIGMENT CONTENT OF GRAPEFRUIT PULP.

Variety	Lycopene	β-carotene	ζ-carotene	Phytofluene
	μg./100 gm.	*μg./100 gm.*	*μg./100 gm.*	*μg/100 gm.*
Texas Pink	237	207	18	5
Red Blush	176	142	10	20
Coachella Pink	28	129	14	3
Coachella White	....	trace	6	9

lycopene, with lesser amounts of ζ-carotene and phytofluene. Traces of α- and γ-carotenes and minor traces of other components were noticed. Lycopene and β-carotene were conspicuously lacking in the white strain. A pale yellow component with an adsorbability close to that of lycopene, (absorption maxima 445, 428 mμ in petroleum ether), ζ-carotene and phytofluene comprised the pigment complex.

The sporting of Marsh White to Marsh Pink involves the emergence of β-carotene as the major pigment with lesser amounts of lycopene. The second mutation, from Marsh Pink to Ruby, establishes lycopene as of even greater significance in the pigment complex. Neither ζ-carotene nor phytofluene show fluctuations of the magnitude necessary to assume that they are intermediates in lycopene or carotene syntheses. We have been informed that the pink strains are noticeably less colored when grown near the coast, and more deeply colored in the hot interior valleys, in Coachella or Arizona. The ratios for lycopene, β- and ζ-carotenes are 1.14 : 1 : 0.09 for Texas Pink from the Rio Grande Valley, compared with 1.24 : 1 : 0.07 for Arizona Red Blush from Mesa, to be contrasted with 0.22 : 1 : 0.11 for the Coachella Pink. These ratios clearly reflect genetic differences in pigmentation between Ruby

and Marsh Pink. However, since the number and nature of the gene differences is unknown in these mutants of Marsh White the evidence cannot yet be interpreted to favor any particular hypothesis as to the mechanism of synthesis of β-carotene or lycopene.

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NOTES

The retiring editor of PLANT PHYSIOLOGY takes this occasion to express appreciation to present and former associate editors and officers of the society for their very helpful and generous counsel over a long period of years in reference to manuscripts and the operation of the journal. It is a pleasure to acknowledge also a debt of gratitude to the present and former secretaries of the American Society of Plant Physiologists, the secretary of the editorial board and to the editor's personal secretary for their able and ready assistance in connection with the editorial work of the journal. The extraordinary cooperation of authors, members, subscribers, and of the society's printer in the interest of high standards have contributed immensely to the usefulness and scientific repute of PLANT PHYSIOLOGY.—W. F. LOEHWING.

A.S.P.P. Election.—The results of the annual election of the American Society of Plant Physiologists for 1953–54 are as follows: President, DR. THEODORE C. BROYER, University of California, Berkeley, California; Vice-President, DR. ALDEN S. CRAFTS, University of California College of Agriculture, Davis, California; Secretary, DR. AUBREY W. NAYLOR, Duke University, Durham, North Carolina; Member of Executive Committee, DR. KENNETH V. THIMANN, Harvard University, Cambridge, Massachusetts; Members of the Editorial Board, DR. DANIEL I. ARNON, University of California, Berkeley, California; and DR. ROBERT H. BURRIS, University of Wisconsin, Madison, Wisconsin.

New England Section.—This section held its annual meeting under the Chairmanship of Dr. J. J. Lyon at Dartmouth College, Hanover, New Hampshire, on May 1–2. Twenty-three papers were presented by the visitors. Fifteen institutions were represented, two of them from Canada. The new officers elected are Chairman, KENNETH E. WRIGHT, Smith College; Vice-Chairman, KENNETH V. THIMANN, Harvard University; and Secretary-Treasurer, LINUS H. JONES, University of Massachusetts.

Olenus Lee Sponsler.—Olenus Lee Sponsler, who was called in 1922 to the University of California at Los Angeles and became emeritus in 1948 after having served as professor of botany for 20 years, died on March 14, 1953, at his home in Sherman Oaks.

Professor Sponsler was born on June 17, 1879, in Akron, Ohio, and began his professional career as a forester. Having received the Bachelor's degree from the University of Michigan in 1910, he served as Adjunct Professor of Forestry and Botany, and Associate Professor in charge of Forestry from 1910–1912 at the University of Nebraska. During this period he studied

with Professor Bessey and received the Master's degree in 1912. He then returned to Michigan as Associate Professor of Forestry from 1912–1917, teaching dendrology, silvics and silviculture, and during this period was in charge of the ecological survey of the Ecological Survey of the Michigan State Geological and Biological Survey. During the first World War he served as wood technologist of the Army Airplane Division of the U. S. Forest Service, engaged in the investigation of plywood for airplane construction. His earliest publications were accordingly concerned with the native trees of the Middle West, and the wood they produce. He was a member of the State Board of Forestry of Wisconsin and of the Society of American Foresters. Thus, at 40 years of age, a distinguished career as a forester lay open to him. In 1919 he elected a new career which should combine his experience in the physical and biological sciences, and enrolled as a graduate student at Stanford University. Studying there with Professors Peirce and Webster, he used the technique of X-ray diffraction to explore the structure of starch, and was awarded the doctorate in 1922 for a thesis entitled "The structure of the starch grain." After coming to Los Angeles, he published a series of studies on the structure of cellulose which attracted international attention because of the concept of long chain molecules which it involved. This work laid the foundation of a second promising career, had he chosen to follow it, because of its application to the then embryonic fiber and textile industries. But beyond cellulose lay that which gives content and form to it: the living substance; and Sponsler chose to address himself to this. He outlined the problem in 1931 in a general statement on the mechanism of cell wall formation, and in 1934 in his Faculty Research Lecture "Living Matter: a Molecular Approach." Thereafter he explored many paths and byways which led toward his objective, and tested his ideas from time to time in symposia and seminars. They began to crystallize only during recent years, and most of this later work, and particularly his thought on electrical properties of protoplasm at molecular and lower micellar levels, is as yet unpublished. Two papers, now in press, written jointly with his colleague, Dr. Jean Bath, are concerned with submicroscopic components of protoplasm revealed by the electron microscope, and shortly before his death, he had reason to believe that he might be viewing for the first time the patterns made by the polypeptide chains themselves within minute protoplasmic particles at the micellar-molecular borderline. His last effort was directed toward a verification of this belief. Thus, his quest of the ultimate beyond the receding horizon led Professor Sponsler from the habit and structure of the trees amongst which he roamed as a youth, into the very core and function of living matter.

Professor Sponsler was awarded a Charles Reid Barnes honorary life membership by the American Society of Plant Physiologists in 1942.—Carl Epling and Karl C. Hamner.

The Yeasts. A Taxonomic Study.—J. Lodder and N. J. W. Kreger-van Rij. Interscience Publishers, Inc., 250 Fifth Avenue, New York 1, New York. 713 pages, 1952. $19.80.

This imposing volume, based largely on the three earlier volumes by Lodder (1934 and 1942) and Stelling-Decker (1931) brings together under one cover the contents of the earlier works and incorporates the results of the intensive studies of the yeasts in recent years so far as they affect the taxonomic treatment. Three families are recognized: The Endomycetaceae, including all ascosporogenous forms, the Sporobolomycetaceae, including the anascosporogenous forms which form ballistospores and the Cryptococcaceae, including all other anascosporogenous forms. These families are divided into subfamilies, tribes and genera largely on morphological characters. Species distinctions emphasize physiological aspects, as in current bacteriological methods. The result is that many of the strains regarded as species would be relegated to lower taxa by other students, but this, of course, in no way detracts from the usefulness of the work for mycologists and greatly increases its value for the majority of those who will use it. Each species is described in orderly sequence and all species are illustrated by drawings of sporulating cells and associated structures and many by drawings from culture slides.

The authors admit that an answer to the question: "What are yeasts?" cannot yet be given. In view of the contemporary interest in these organisms, it may be expected that a provisional answer may emerge in the near future. The volume under consideration affords an indispensable starting point for further work.

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Manuscripts for Plant Physiology should be addressed to Prof. David R. Goddard, Botanical Laboratories, University of Pennsylvania, Philadelphia, Pennsylvania. Drawings and photographs should not exceed 8.5 × 11 inches in size, should be numbered correctly on the back, and separate typewritten legends included with the text of the manuscript. Tables and cuts should be used sparingly, and the text presented in concise form. Instructions to authors for preparation of manuscripts are given in the January number, Volume 28, 1953, of Plant Physiology, 149–150.

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